Mervi Haapsamo

LOW-DOSE ASPIRIN THERAPY IN IVF AND ICSI PATIENTS
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IN IVF AND ICSI PATIENTS

Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in Auditorium 4 of Oulu University Hospital, on 9 December 2011, at 12 noon

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Abstract
The first aim of this randomized, placebo-controlled and double-blind study was to investigate whether low-dose aspirin therapy, started prior to controlled ovarian hyperstimulation, improves ovarian stimulation response, uterine haemodynamics and clinical pregnancy rates in unselected patients who underwent in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). The second aim was to examine if the maternal serum placental proteome is different in IVF/ICSI pregnancies compared with spontaneous pregnancies, and whether low-dose aspirin modifies maternal serum placental protein expression and uteroplacental haemodynamics during the first half of pregnancy. Finally, the effect of low-dose aspirin therapy on the incidence of hypertensive pregnancy complications among women who became pregnant after IVF/ICSI was investigated.

Low-dose aspirin therapy did not increase the number of oocytes retrieved, the total number of embryos or number of top-quality embryos, endometrial thickness or uterine haemodynamics on the day of embryo transfer (ET) or clinical pregnancy rates compared with placebo-treated IVF/ICSI women. On the day of ET, low-dose aspirin did not affect UtA vascular impedance, but the incidence of non-optimal uterine artery haemodynamics (UtA PI ≥ 3.0) was statistically significantly lower (p < 0.05) in the aspirin group compared with the placebo group. In the placebo-treated IVF/ICSI patients, maternal serum proteome analysis showed altered protein expression compared with women with spontaneous pregnancies. Between aspirin- and placebo-treated IVF/ICSI patients, proteome analysis showed a unique and distinct pattern of differentially expressed proteins including extra-cellular matrix, complement and transport proteins. At 6 weeks’ gestation, arcuate artery PI and at 18 weeks’ gestation, UtA PI values were lower (p < 0.05) in the aspirin group than in the placebo group.

In conclusion, low-dose aspirin therapy, when started concomitantly with controlled ovarian hyperstimulation, did not improve ovarian responsiveness, uterine receptivity, pregnancy outcome in unselected IVF/ICSI women or affect UtA vascular impedance on the day of ET. Low-dose aspirin modified the early placentation process and reduced uteroplacental vascular impedance in mid-pregnancy, but did not decrease the incidence of hypertensive pregnancy complications.

Keywords: antiplatelet therapy, assisted reproduction, Doppler ultrasonography, ovarian responsiveness, placenta-related pregnancy complications, placentation, pregnancy rate, uterine haemodynamics
Haapsamo, Mervi, Matala-annoksinen aspiriini IVF ja ICSI potilailla
Oulun yliopisto, Lääketieteen tiedekunta, Kliinisen lääketieteen laitos, Synnytys- ja naistentaudin, Biolääketieteen laitos, Fysiologia PL 5000, 90014 Oulun yliopisto; Itä-Suomen yliopisto, Terveystieteiden tiedekunta, Kliinisen lääketieteen yksikkö, Synnytys ja naistentaudin, PL 1627, 70211 Kuopio; Tampereen yliopisto, Lääketieteen yksikkö, Tampereen yliopistollinen sairaala, Synnytys ja naistentaudin, 33014 Tampereen yliopisto; Väestöliiton lapsottomuusklinikka, Kiviharjuntie 11, 90220 Oulu; Terveystieteiden yliopisto, Lastentaudit, BTE355 3181 S.W Sam Jackson Park Road, 97239 Portland, USA

Tiivistelmä

Tämän satunnaisetetun ja plasebo-kontrolloidun kaksoissokkotutkimuksen tavoitteena oli tutkia keinoalkuisia hedelmöityshoitoja saavilla naisilla matala-annoksisen ASA-hoidon (100 mg/vrk) merkitystä munasarjojen stimulaatiovasteeseen, alkion kiinnittymiseen, istukan muodostumiseen ja kehittymiseen sekä lääkkeiden vaikutusta kohdun, istukan ja sikiön verenkiertoon, kun lääkisy aloitettiin munasarjojen stimulaatiohoidossa. Lisätavoitteena oli selvittää, onko plasebo-munasarjoja saavien naisten raskauksissa todetut tavat merkillävissä istukkaproteomiikalla kehittyvät vähemmän kymmenen prosenttia vähemmän. Toisena lisätavoitteena oli selvittää, onko lapsettomuushoidojen saavatut naisten raskauksissa todettavissa proteinien esiintyvyydessä eri raskauskunttoihin käytetty matala-annoksinen ASA-hoidon vaikutusta. Toisena lisätavoitteena oli selvittää, onko hidastuneeseen pre-eklampsiaan liitettyyn raskauden päättymiseseuran tila.

Matala-annoksinen asetyylisalisyylihappo (ASA) ei paranna keinoalkuisia hedelmöityshoitojen hoitotuloksia eikä vaikuta kohdun verenkiertoon tai kohdun limakalvon paksuuteen ultraäänlä arvioiduna. Matala-annoksista ASA-hoidoa käyttäneiden potilaiden raskauttaa todettiin kuitenkin merkitsevästi vähemmän naisia, joilla oli huonoa hoitotulosta keinoalkuisissa hedelmöityshoidoissa ennakkoista korkea moleminpuolinen kohtuvalmioiden verenvirtausvarastus alkion sirrottavien räät vallanista raskaaksi tulleiden naissiin. Tämä todettiin matala-annoksista ASA-hoidoa, toidinpaineen kohdun verenvirtausvaatimus matelamaksi alkion- ja keskiraskaudessa raskaaksi tulleiden naissiin. Matala-annoksinen asetyylisalisyylihappo (ASA) vähentää raskauksissa oireihin liittymisen epätasapainoa, jota voi vaikuttaa matala-annoksista ASA-hoidolla. Tampereen yliopistollinen sairaala, Synnytys ja naistentaudin, 33014 Tampereen yliopisto; Väestöliiton lapsottomuusklinikka, Kiviharjuntie 11, 90220 Oulu; Terveystieteiden yliopisto, Lastentaudit, BTE355 3181 S.W Sam Jackson Park Road, 97239 Portland, USA

Asiasanat: Doppler ultraäänä, istukan kehitys, istukkaperäiset raskauskomplikaatiot, keinohedelmöitys, kohdun verenkierto, munasarjojen hormonivaste, raskauskulu
In loving memory of my Mom
Acknowledgements

The research for this thesis was carried out at the Departments of Obstetrics and Gynaecology of Oulu, Tampere and Kuopio University Hospitals, at the Infertility Clinic of the Family Federation of Finland, Oulu and at the Department of Pediatrics, Oregon Health Sciences University, during the years 2001–2007.

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Rovaniemi, October, 2011

Mervi Haapsamo
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ang</td>
<td>Angiopoietin</td>
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<tr>
<td>C</td>
<td>Complement</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CSH1</td>
<td>Chorionic somatomammoprotein 1</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase enzyme</td>
</tr>
<tr>
<td>DA</td>
<td>Ductus arteriosus</td>
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<tr>
<td>DIGE</td>
<td>Difference gel electrophoresis</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>eSET</td>
<td>Elective single embryo transfer</td>
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<tr>
<td>ET</td>
<td>Embryo transfer</td>
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<tr>
<td>(s)flt-1</td>
<td>(soluble) Fms-like tyrosine kinase 1, (soluble) vascular endothelial growth factor receptor 1</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>GdA</td>
<td>Glycodelin A</td>
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<tr>
<td>GnRH</td>
<td>Gonadotrophin-releasing hormone</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotrophin</td>
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<tr>
<td>HIF-1α</td>
<td>Hypoxic-inducible factor-1 alpha</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<tr>
<td>ICSI</td>
<td>Intracytoplasmic sperm injection</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor-binding protein</td>
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<tr>
<td>IGFR</td>
<td>Insulin-like growth factor receptor</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>INT-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>IPG</td>
<td>Immobilized pH gradient</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth restriction</td>
</tr>
<tr>
<td>IVF</td>
<td><em>In vitro</em> fertilization</td>
</tr>
<tr>
<td>KDR</td>
<td>Kinase insert domain receptor, VEGFR2</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MI</td>
<td>Mechanical index</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PAPP-A</td>
<td>Pregnancy-associated plasma protein A</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial adhesion molecule</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PI</td>
<td>Pulsatility index</td>
</tr>
<tr>
<td>PIH</td>
<td>Pregnancy-induced hypertension</td>
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<tr>
<td>PLGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>PP13</td>
<td>Placental protein 13</td>
</tr>
<tr>
<td>PSG1</td>
<td>Pregnancy-specific β1 glycoprotein 1</td>
</tr>
<tr>
<td>RI</td>
<td>Resistance index</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TI</td>
<td>Thermal index</td>
</tr>
<tr>
<td>Tie-2</td>
<td>Angiopoietin receptor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrix metalloproteinases</td>
</tr>
<tr>
<td>uNK</td>
<td>Uterine natural killer cell</td>
</tr>
<tr>
<td>UtA</td>
<td>Uterine artery</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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</table>
List of original articles

This thesis is based on the following articles, which are referred in the text by their Roman numerals.


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1 Introduction

Poor ovarian and endometrial responses to controlled ovarian hyperstimulation in assisted reproduction techniques can lead to decreased pregnancy rates. The main factors that affect the outcome of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are the number of oocytes retrieved, quality of the embryos, endometrial receptiveness and age of the woman (Weckstein et al. 1997, Ebner et al. 2000, Terriou et al. 2001). Controlled ovarian hyperstimulation has potential health risks and thus it is important to find factors that may improve the ovarian response to gonadotrophins, the endometrial environment for embryo implantation and maximise pregnancy rates.

A favourable endometrial milieu for embryo transfer has been predicted noninvasively by ultrasonography by measuring blood flow velocities and blood flow velocity waveform indices in the uterine, subendometrial and endometrial arteries (Zaidi et al. 1996, Chien et al. 2002, Merce et al. 2008) and by assessing endometrial thickness, morphology and volume (Zaidi et al. 1995, Yaman et al. 2000, Schild et al. 2001). Women with successful implantation after IVF and ICSI have shown more optimal uterine blood flow and thicker endometria than women with implantation failure (Noyes et al. 1995). In particular, increased uterine artery (UtA) vascular impedance and low endometrial blood flow during embryo transfer have been associated with poor implantation and pregnancy rates (Coulam et al. 1994, Zaidi et al. 1995, Cacciator et al. 1996, Chien et al. 2002). However, some results have not revealed such an association (Isakkson et al. 2003, Puerto et al. 2003).

In IVF and ICSI pregnancies, maternal serum placental protein levels are different from those observed in spontaneously conceived pregnancies (Liao et al. 2001, Perheentupa et al. 2002, Hui et al. 2005, Tul & Novak-Antolic 2006, Anckaert et al. 2008). The aetiology is unknown. However, altered maternal serum placental protein concentrations in the first and second trimesters are associated with increased risks of pre-eclampsia and/or fetal intrauterine growth restriction (IUGR) later in pregnancy (Sebire et al. 2000, Tul et al. 2003). In pregnancies complicated by pre-eclampsia and IUGR, placental histology shows a superficial invasion of trophoblastic cells that is thought to lead to insufficient remodelling of maternal spiral arteries and abnormal development of placental vasculature (Brosens et al. 1972, Roberts et al. 1989, Lim et al. 1997b, Zhou et al. 1997). Abnormal maternal serum placental protein levels have been related to increased uteroplacental vascular impedance in mid-gestation, reflecting deficient spiral artery transformation during
the early placentation period and thus increasing the risk of pre-eclampsia and IUGR (Albaiges et al. 2000, Papageorghiou et al. 2001). Furthermore, in pre-eclampsia the production of thromboxane $A_2$, which induces platelet aggregation and vasoconstriction, is excessive (Makila et al. 1984, Wang et al. 1992).

Low-dose acetylsalicylic acid (aspirin) irreversibly inhibits the enzyme cyclo-oxygenase in platelets, preventing the synthesis of thromboxane $A_2$ (Vane 1971, Willis 1974). By way of this mechanism, low-dose aspirin may enhance uterine and ovarian blood flow and tissue perfusion and improve oocyte yield, quality of embryos as well as the endometrial milieu for implantation, and thus increase pregnancy rates. By correcting the imbalance between thromboxane $A_2$ and prostacyclin production, low-dose aspirin therapy could theoretically improve trophoblast invasion into maternal spiral arteries and decrease the incidence of hypertensive pregnancy complications.

In this randomized, placebo-controlled and double-blind study, the effect of low-dose aspirin, when started prior to conception, was examined in connection with ovarian responsiveness, uterine receptivity on the day of embryo transfer and pregnancy outcome in unselected IVF and ICSI patients. In pregnant IVF and ICSI women we investigated whether IVF/ICSI alters the maternal serum placental protein pattern, in comparison with women with spontaneous pregnancies, and if placentation is modified by low-dose aspirin therapy. In addition, we examined whether low-dose aspirin therapy improves utero- and umbilicoplacental haemodynamics during the first half of pregnancy.
2 Review of the literature

2.1 Embryo implantation and early placentation

The implantation process involves complex and synchronized molecular and cellular events between the endometrium and the implanting embryo. The trophoblast cells, i.e. the peripheral part of the blastocyst, produce several signalling factors, such as cytokines and growth factors, enabling invasion into maternal uterine endometrium. In order to achieve successful implantation, the endometrium must undergo the process of decidualization, in which endometrial stromal cells, uterine glands and vessels, as well as uterine immune cells are modified. In humans, decidualization begins in the late secretory phase of the menstrual cycle and is independent of the presence of the blastocyst in the uterine cavity. Decidualization continues in early pregnancy and it is thought to regulate subsequent trophoblast cell differentiation, invasion and placental formation (Lunghi et al. 2007).

2.1.1 Trophoblast differentiation and migration

During the implantation process trophoblasts differentiate into two major cell lineages, syncytiotrophoblasts and invasive cytotrophoblasts. Syncytiotrophoblast cells are villous trophoblasts that constitute the outer layer of floating villi. They are in direct contact with the intervillous space and are therefore responsible for placental nutrient and gas exchange. Invasive trophoblasts are called extravillous trophoblasts and they can be defined as interstitial and endovascular. A schematic diagram of extravillous trophoblast differentiation, migration and invasion into the myometrium and maternal spiral arteries is presented in Figure 1. In early pregnancy these extravillous trophoblast cells begin to proliferate and form cytotrophoblastic anchoring cell columns from which the invasive trophoblasts emanate. The interstitial invasive trophoblasts migrate to the maternal side and invade the uterine stroma anchoring the placenta to the uterine wall. They arrive in the inner myometrium at around 8 weeks of gestation (Pijnenborg et al. 1981). Invading trophoblasts subsequently fuse into multinuclear giant cells, and this process is thought to slow down, and ultimately stop the invasion at the level of the inner myometrium (Pijnenborg 1996a). Endovascular invasive trophoblasts migrate towards maternal spiral arteries and invade the endothelial cells of these vessels (Hamilton & Boyd 1966, Harris & Ramsey 1966).
2.1.2 Endovascular trophoblast invasion

Endovascular cells in maternal spiral arteries were first described by Friedländer in 1870, but in the 1920s these cells were believed to be of trophoblastic origin (Grosser 1927). In 1966 Hamilton and Boyd confirmed this finding by demonstrating histological continuity of cytotrophoblasts from the cell columns of anchoring villi (Hamilton & Boyd 1966). Anatomically, spiral arteries extend through the distal myometrium to the endometrial surface and supply blood to the upper functional layer of the endometrium. During pregnancy spiral arteries open into the intervillous space to flush the syncytiotrophoblast layer (Figure 1). In early pregnancy, spiral arteries in the placental bed undergo structural changes that ensure adequate blood flow to the placenta during later stages of pregnancy.

Endovascular invasion is suggested to occur by two different anatomical pathways (Figure 1) (Ramsey & Harris 1966, Pijnenborg 1990, Kaufmann et al. 2003). The intravasation model proposes that endovascular trophoblast cells represent differentiated interstitial trophoblast cells. According to this model, the trophoblasts invade the arterial wall from the surrounding stromal zone and migrate inside the lumen through the arterial wall. The extravasation model, which is based on animal studies, suggests that trophoblast cells gain access to the spiral artery lumen via the entrance to the intervillous space. Thereafter, the cells migrate along the arterial lumen, retrograde to blood flow, adhering to the endothelium. After invading the maternal spiral arteries, endovascular trophoblasts displace and replace the endothelial cell lining and play an important role in the degradation of muscle and elastic tissue of the arteries. This process is called “physiologic transformation” of spiral arteries and it is part of normal pregnancy. These structural changes, particularly replacement of the muscular cell layer in the media by fibrinoid material, lead to reduced responsiveness of these vessels to vasoconstricting agents, to loss of vasomotor control and finally to dilatation of the arterial lumen (Brosens et al. 1967). As a result of this transformation, spiral arteries are low resistance and high capacitance vessels, in order to maximize the delivery of maternal blood to the intervillous space of the placenta.

Trophoblast invasion of spiral arteries has been suggested to occur in two stages (Robertson et al. 1975, Pijnenborg et al. 1983). In the first trimester, the invasion takes place in the decidual segments of spiral arteries and the transformation of myometrial segments occurs in the second trimester. However, this proposal has been criticized on the basis of lack of morphological evidence (Robson et al. 2001). The results of recent studies have suggested that trophoblast invasion of spiral
arteries is a continuous process, which is normally completed by 18–22 weeks of gestation (Pijnenborg et al. 1981).

Fig. 1. Schematic diagram of trophoblast differentiation, migration and endovascular invasion. Endovascular trophoblast invasion into maternal spiral arteries by way of intravasation (a) and extravasation models (b). (Modified from Lunghi et al. 2007).

2.2 Regulation of implantation and placentation

Implantation and placentation lead to trophoblast-mediated remodelling of the uterine endometrium and vasculature and to the establishment of feto-maternal interactions. The outer layer of the blastocyst, trophectoderm and maternal decidua
have specific attachment and invasive properties that are essential for implantation and further maintenance of pregnancy. The most important currently known regulatory factors presented in this section are summarized in Table 1.

### 2.2.1 Oxygen tension and HIF-1α

In normal pregnancies, a relatively low oxygen environment characterizes blastocyst implantation, early placentation and embryonic development until the 10\textsuperscript{th} gestational week (Jauniaux \textit{et al.} 1991, Jaffe & Woods 1993, Coppens \textit{et al.} 1996). The intervillous circulation is established peripherally at around 9 weeks of gestation and expands progressively to encompass the whole placenta after 12 weeks of gestation (Jauniaux \textit{et al.} 2003). During the first trimester, the intervillous space is separated from the uterine circulation by trophoblast cell plugs that occlude the tips of spiral arteries (Hustin & Scaaps 1987) (Figure 1). This physiological hypoxia protects the developing embryo against the deleterious and teratogenic effects of free oxygen radicals. Moreover, recent evidence also indicates that hypoxia is necessary to maintain stem cells in a fully pluripotent state (Forristal \textit{et al.} 2010). Limited blood flow in the intervillous space during the first trimester has been documented by Doppler ultrasonographic and anatomical studies (Jauniaux \textit{et al.} 2003). At the end of the first trimester, these plugs are progressively dislocated, allowing increased maternal blood supply into the intervillous space.

In early pregnancy complications, the placenta appears to be hypervascularized in colour flow mapping by Doppler ultrasonography, suggesting that plugging of the spiral arteries is incomplete (Jaffe \textit{et al.} 1992, Kurjak & Kupesic 1997). This exposes syncytiotrophoblasts to excess maternal blood supply and thus high oxygen levels. In early pregnancy, placental tissue contains low concentrations of antioxidant enzymes, thus being highly vulnerable to oxygen-mediated cell damage. Premature and widespread onset of maternal blood flow in the intervillous space has been associated with superficial trophoblast invasion and, further, early pregnancy loss or pre-eclampsia (Jaffe & Warsof 1991, Jauniaux \textit{et al.} 2003, 2006).

A hypoxic environment in early pregnancy stimulates hypoxic-inducible factor-1 alpha (HIF-1α), which promotes cytotrophoblastic proliferation (Genbacev \textit{et al.} 1997) and villous vasculogenesis (Charnock-Jones & Burton 2000). Expression of HIF-1α falls at around 10 weeks of gestation, when placental oxygen tension starts to increase. If the oxygen tension fails to increase or trophoblasts do not detect this increase, HIF-1α levels remain high, resulting in superficial trophoblast invasion, and increasing the risk of pre-eclampsia (Caniggia \textit{et al.} 2000a).
2.2.2 Growth factors and their receptors

Angiogenic factors

A number of growth factors and their receptors are expressed in the human placenta. Vasoactive growth factors that promote and regulate vasculogenesis (formation of new vessels) and angiogenesis (formation of new capillaries from pre-existing vessels) are indispensable in the placenta formation process. One of the most essential factors for placental and embryonic development is vascular endothelial growth factor A (VEGF-A, VEGF). In the human placenta, VEGF is expressed in the villous trophoblast and stromal macrophages (Jackson et al. 1994, Clark et al. 1998). The most important regulator of VEGF is hypoxia, which induces VEGF expression via HIF-1 (Semenza 2000) and other transcription factors (e.g. early growth response 1 and translation elongation factor 1) (Yan et al. 2000, Shie et al. 2004). Secreted VEGF mediates its actions via two endothelial tyrosine kinase receptor isoforms, flt-1 (fms-like tyrosine kinase 1, VEGFR1) and KDR (kinase insert domain receptor, VEGFR2). Binding to VEGFR-2 causes endothelial cell differentiation and proliferation (Bernatchez et al. 1999), whereas binding to VEGFR-1 mediates endothelial cell interaction and tube formation (Fong et al. 1995). In addition to the transmembrane form, VEGFR-1 also has a soluble isoform (sflt-1), which acts as a potent antagonist of VEGF and placental growth factor (PIGF). Therefore, sflt-1 reduces free circulating concentrations of VEGF and PIGF and may contribute to the pathogenesis of pre-eclampsia by inhibiting physiological vasodilatation. VEGF also increases nitric oxide production, which induces vasodilatation (Hood et al. 1998, Otrock et al. 2007).

While VEGF production reaches its peak at the end of the first trimester, the expression of PIGF is low during early pregnancy and increases towards term. The physiological role of PIGF in placental development is unclear. PIGF-deficient mice do not show any signs of abnormal placental development during pregnancy (Charnock-Jones & Burton 2000).

In addition to the VEGF family, angiopoietin-1 (Ang-1) and its antagonist angiopoietin-2 (Ang-2) play a role in angiogenesis and vascular remodelling in early human placenta (Rowe et al. 2003). They cause branching and non-branching angiogenesis, but do not participate in the formation of a primitive vascular network from endothelial progenitor cells. Ang-1 causes endothelial maturation and vascular stabilization, whereas Ang-2 promotes angiogenesis in the presence of VEGF (Suri et al. 1996, Maisonpierre et al. 1997, Thurston 2003). Both growth
factors act via Tie-2 (angiopoietin-2) receptor. In women with pre-eclampsia, serum concentrations of Ang-2 have been shown to be significantly lower than in women with uncomplicated pregnancies (Hirokoshi et al. 2005).

Transforming growth factors

Transforming growth factors (TGFs) consist of two different types of polypeptide growth factors, TGF-α and TGF-β. These TGFs are not structurally or genetically related and act through different receptor mechanisms. While TGF-α is involved in oncogenic transformation, TGF-β superfamily members are produced at the feto-maternal interface and are major factors regulating trophoblast invasion in the uterus (Lysiak et al. 1995, Caniggia et al. 2000b). Specifically, they are involved in cell proliferation and promote a change in integrin expression by down-regulation of laminin receptor and up-regulation of fibronectin receptor. Of three isoforms, TGF-β3 expression is high in early pregnancy and it falls precipitously around 10 weeks of gestation in parallel with HIF-1α expression, when placental oxygen tension increases and maximal trophoblast invasion occurs (Caniggia et al. 2000b). TGFs inhibit extravillous trophoblast differentiation to a non-proliferative, invasive phenotype. Inhibition of TGF-β3 (Caniggia et al. 1999), or down-regulation of the TGF-β receptor, endoglin (Caniggia et al. 1997), induces trophoblast differentiation.

TGF-β biological function is also regulated by vasorin, which is a TGF-β-binding protein. Vasorin acts as an inhibitor of TGF-β signalling by binding directly to it, and thus promoting trophoblast differentiation in an invasive direction (Ikeda et al. 2004). Activin and inhibin are glycoprotein hormones that belong to the TGF-β protein superfamily. During pregnancy, they and their receptors are predominantly secreted by the placenta. Activin A is also secreted from decidua and developing embryo. It promotes decidualization and implantation and stimulates hormone production from cytotrophoblasts (Caniggia et al. 1997). Moreover, it enhances cytотrophoblast column formation (Caniggia et al. 1997) and differentiation to an invasive phenotype by increasing the secretion of other molecules involved in blastocyst implantation, such as matrix metalloproteinases (MMPs) and leukaemia inhibitory factor (LIF), which is an anti-inflammatory cytokine (Jones et al. 2002, 2006). In cytotrophoblast cells, activin stimulates the secretion of hCG and progesterone, acting as an important local regulator of placental development and function (Petraglia et al. 1989). The effects of activin A are hindered by follistatin, activin-binding protein and inhibin. Inhibin is expressed only in the villous trophoblasts, not in endometrial components. It inhibits steroidogenesis
and production of hCG by the cytotrophoblasts. It is considered to be a particularly specific marker of early placentation development, and is detectable from 14 days post-embryo transfer following IVF, indicating its potential as an early marker of IVF success (Birdsall et al. 1997). Conversely, a low level of inhibin A in early pregnancy is indicative of pregnancy failure, and several studies have shown a clear correlation between low inhibin A levels and subsequent miscarriage (Wallace et al. 2004, Prakash et al. 2005). Both activin A and inhibin A are detectable in fusing syncytiotrophoblast and syncytialization (Debieve et al. 2000, Jones et al. 2006).

Insulin-like growth factor superfamily

Insulin-like growth factors (IGFs) are potent stimulators of tissue growth and they regulate metabolic state, mitogenesis, differentiation and cell survival (Jones & Clemmons 1995, Vincent & Feldman 2002). Human fetal tissues and the placenta express IGF-I and IGF-II from early gestation onwards. IGFs have been shown to be important in the regulation of fetal weight gain. Partial deletion in the coding region of the human IGF-I gene results in severe intrauterine growth restriction (Woods et al. 1996), and IGF levels correlate with birth weight (Westwood et al. 2001). Moreover, IGF-II-null mice have small placentas, indicating that IGFs may influence fetal growth by promoting placental growth (Roberts et al. 2008). Experimental reduction in either IGF-I or IGF-II levels results in decreased proliferation and survival of placental fibroblasts (Miller et al. 2005). IGF-I and IGF-II mediate their effects by binding primarily to the type 1 IGF receptor (IGF1R). Recent studies have shown that IGF-I and IGF-II regulate the placentation process by enhancing cytotrophoblast proliferation and syncytial formation, and they can prevent trophoblasts from undergoing apoptosis (Forbes et al. 2008). Notably, IGF-II is known to be involved in trophoblast invasion and thus it may promote spiral artery recruitment in the placenta (Irving & Lala 1995).

The bioavailability and biological actions of IGFs are regulated by a family of six high-affinity binding proteins (IGFBPs 1-6) (Clemmons 1997). Binding of IGFs to IGFBPs can result either in inhibition or enhancement of their actions. This depends, at least in part, on whether the affinity of IGFBP for IGFs is greater than that of the IGF1R. Preferential binding of IGFs to IGFBPs could then reduce their availability for the receptor. Binding of IGFBPs to the cell surface or extracellular matrix reduces their affinity for IGFs. IGFBPs may enhance the action of IGFs by maintaining a local pool of IGFs that is available for receptor binding. Several
different types of proteases cleave IGFBPs, resulting in complete or partial reduction of their availability for IGF-I and -II.

Secretory phase endometrium produces IGFBP-1 to -4 and -6. In first trimester placenta, IGFBP-1 is first expressed in the epithelium of the endometrial glands and in some decidualized stromal cells. However, from 12 weeks of gestation onwards, IGFBP-1 is expressed exclusively in the decidual cells (Han et al. 1996). In the second and third trimesters, decidual cells of the basal plate region express IGFBP-1 to -6, with IGFBP-1 being the most abundant. However, not all decidual cells express IGFBP equally. IGFBP-1, -2, -4 and -6 are expressed in the majority of decidual cells, whereas IGFBP-3 and -5 are expressed in a selected population of cells. Non-decidualized endometrial stromal cells express only IGFBP-5 (Han et al. 1996). Since human decidua produces IGFBP-1 to -6, and the extravillous trophoblasts of the basal plate produce IGF-II, it has been proposed that there is an interaction between IGF-II and its binding proteins at the maternal-fetal interface during trophoblast invasion and decidualization (Nonoshita et al. 1994, Irwin & Giudice 1998, Giudice et al. 2002). Furthermore, IGFBP-1 peptide contains a recognition site for a number of cell adhesion molecules, including α5 integrin. This integrin component is known to be expressed by invading and non-invading trophoblasts at the maternal-fetal interface (Damsky et al. 1992, Zhou et al. 1993a, 1993b, 1997, Irwin & Giudice 1998). As first shown in Chinese hamster ovary cells and porcine vascular smooth muscle cells, IGFBP-1 can bind to α3β1 integrin of trophoblasts and either stimulate their motility (Gleeson et al. 2001) or inhibit trophoblast invasion into decidualized stromal cells (Irwin & Giudice 1998), depending on the binding site of IGFBP-1.

2.2.3 Immune system

Successful pregnancy remains to some extent an immunological enigma; the fetus inherits histocompatibility antigens from the father and yet coexists within the mother’s uterus in harmony throughout pregnancy. Villous trophoblasts are in contact with the maternal circulating blood in the intervillous space from 10 weeks of gestation onwards. Leukocytes are an important component of the human uterine decidua in normal pregnancy and are believed to have an essential role in recognition of semiallogenic fetal cells.
**Uterine natural killer cells**

The most abundant and best investigated form of leukocyte at the maternal-fetal interface is the uterine natural killer (uNK) cell. These cells are granulated lymphocytes and are different from NK cells in peripheral blood. They are abundant in mid- and late stages of the menstrual cycle, as well as in early pregnancy. While virtually absent at term gestation, uNK cells are suggested to have an important role in implantation and placentation. Croy et al. (Croy et al. 1997) have demonstrated uNK cell aggregation around the spiral arteries in early pregnancy. Carlino et al. (Carlino et al. 2008) found that peripheral blood NK cells from pregnant women had a greater migratory capacity through decidual endothelial cells than peripheral blood cells from males or non-pregnant women. Moreover, the cytotoxic activity of uNK cells seems to be lower than that of peripheral NK cells. Human leukocyte antigen-G (HLA-G) produced by trophoblast cells protects them against the cytotoxic activity of uNK cells (Chumbley et al. 1994, Rouas-Freiss et al. 1997). This suggests that uNK cells mediate decidual reaction, immunoregulation of trophoblast invasion and remodelling of spiral arteries in the decidua.

Uterine NK cells produce cytokines that most likely regulate trophoblast invasion and angiogenesis (Carlino et al. 2008). The cytokines include tumour necrosis factor-α, TGF-β1 and interferon gamma (INF-γ), which inhibit trophoblast invasion (Lash et al. 2005), and interleukin-8 (IL-8) and IL-10, which stimulate trophoblast invasion (Hanna et al. 2006). Uterine NK cells also regulate their actions through inhibition and activation of surface receptors. Inhibition of these receptors appears to block uNK cell cytotoxicity. In NK cells, inhibitory receptors are dominant, thus providing a protective mechanism against NK cell-mediated lysis (Lanier 1998).

Increased numbers of uNK cells in the endometrium and imbalance in expression between inhibitory and stimulatory uNK cell receptors have been associated with recurrent miscarriages (Quenby et al. 1999, Ntrivalas et al. 2005) and recurrent implantation failures after IVF (Ntrivalas et al. 2005, Tuckerman et al. 2007). Furthermore, increased numbers of uNK cells have been reported in deciduas in women with pre-eclampsia (Stallmach et al. 1999, Wileczynski et al. 2003, 2006, Bachmayer et al. 2006) and in pregnancies complicated by IUGR with or without pre-eclampsia (Eide et al. 2006).
Other leukocytes

Macrophages represent the second most abundant leukocyte population in decidua (Bulmer & Johnson 1984, Bulmer et al. 1988). Unlike uNK cells, macrophages are found in both endometrium/decidua and myometrium. In early pregnancy they are present in spiral arteries and glands (Bulmer & Johnson 1984) and extravillous trophoblast (Bulmer et al. 1988). It has been proposed that they play a role in phagocytosis of cell debris produced during implantation (Abrahams et al. 2004), in immunosuppression (Mizuno et al. 1994) and in prevention of maternal T lymphocyte activation (Heikkinen et al. 2003). Macrophages seem to regulate trophoblast migration and invasion. Renaud et al. (Renaud et al. 2005) have demonstrated that activated peripheral blood macrophages inhibit trophoblast invasion, while non-activated macrophages have no effect on trophoblast invasion.

Other important leukocyte populations at the maternal-fetal interface are T lymphocytes, natural killer T (NKT) cells, regulatory T cells and dendritic cells. Normal pregnancy requires a shift from T helper (Th) 1 (cellular immune system) immunity to Th2 (humoral immune system) immunity. If Th1 cytokines (e.g. INF-γ) predominate, trophoblast invasion is inhibited, resulting in failed or compromised pregnancy (Michimata et al. 2003, Saito & Sakai 2003). NKT cells can rapidly produce large quantities of cytokines, specifically INF-γ (Bendelac et al. 1997, Tsuda et al. 2001), which has a role in vascular remodelling in early pregnancy (Croy et al. 1997, Lash et al. 2006). Regulatory T lymphocytes induce a tolerant microenvirnoment at the maternal-fetal interface by producing TGF-βs, Toll-like receptors (membrane proteins that play a key role in innate immunity), IL-6 and IL-10 (Zenclussen et al. 2006a, 2006b), Fas ligand and indoleamine 2,3-dioxygenase (IDO), the enzyme that suppresses T lymphocyte proliferation and activity (Heikkinen et al. 2004). Dendritic cells are part of the innate immune system and are closely related to macrophages. They have been identified in decidua and have been shown to be able to produce IDO and interact directly with HLA-G of trophoblast cells. Moreover, they may prime resting NK-cells and activate them (von Rango 2008).

Complement system

The complement system is an immunological defence system that consists of a number of small proteins found in the blood, normally circulating as inactive precursors. These proteins eliminate antigens and pathogens from an organism and are part of the
innate immune system. The complement cascade can be activated by three distinct biochemical pathways: classical, alternative and lectin. Complement activation results in enzymatic cleavage of complement 3 (C3) protein, which causes the formation of a membrane attack complex. This leads to increased vascular permeability, attraction of leukocytes and their immobilization at the site of inflammation, enhanced phagocytosis and cell lysis (Frank & Atkinson 2001). The complement system can be extremely harmful to host tissue. Thus, its activation must be tightly regulated. Women with antiphospholipid antibodies (aPLs) show complement component aberrations and abnormal complement-mediated reactions (Pierangeli et al. 2005). Antiphospholipid antibodies are known to represent a risk factor of recurrent pregnancy loss and other pregnancy complications (Francis et al. 2006, Tincani et al. 2010). In vitro studies have demonstrated that aPLs have a direct effect on trophoblasts, causing reduced proliferation, hCG secretion, invasiveness and adhesion molecule expression, and increased apoptosis. All these aPL-mediated effects may lead to abnormal placentation, miscarriages and other obstetric complications (Francis et al. 2006, Girardi et al. 2006, 2010, Meroni et al. 2010, Tincani et al. 2010).

**Human leukocyte antigen**

Extravillous trophoblasts express only major histocompatibility complex (MHC) class I molecules (Moffett & Loke 2006). In human pregnancy, expressed molecules include human leukocyte antigens (HLAs) -C, -E and -G (Hiby et al. 1999, King et al. 2000a, 2000b, Moffett & Loke 2006). The classical MHC class I molecules, HLA-A and HLA-B, which initiate allograft rejection, are not expressed in extravillous trophoblasts. Many studies have shown that expression of HLA-G is absent or reduced in pre-eclampsia, suggesting that HLA-G, perhaps with HLA-E, protects invasive trophoblasts from uNK cell attack (Navarro et al. 1999). When invasive trophoblasts lacking HLA-G encounter uNK cells they are destroyed, resulting in superficial invasion of extravillous trophoblast cells into the decidua and abnormal spiral artery remodelling, seen in pre-eclamptic placentas. On the other hand, at the feto-placental site syncytiotrophoblasts express no MHC antigens on the cell surface (Moffett & Loke 2006).

**Adhesion molecules**

Members of the immunoglobulin superfamily, vascular cell adhesion molecules (VCAMs), intercellular adhesion molecules (ICAMs) and platelet endothelial
adhesion molecules (PECAMs), facilitate trophoblast migration and invasion and may hinder neutrophil-mediated vascular cell damage together with other adhesion molecules (i.e. integrins, cadherins and selectins) (Humphries et al. 1995). The key function of adhesion molecules is to promote the firm adhesion of leukocytes to vascular endothelium, and prevent leukocyte extravasation (Springer 1994), but they also act as angiogenic agents (Kwee et al. 1995). VCAM1 is a cytokine-inducible cell surface protein and it is expressed principally on endothelial cells. In placental villous cells, the down-regulated production of VCAM1 towards term indicates that it has a specific role at the developmental stage of pregnancy (Rajashekhar et al. 2003). VCAM1 expression is stimulated by cytokines and it interacts with α4 integrin, which is its only known receptor (Ruegg et al. 1992, Humphries et al. 1995). VCAM1-deficient mice are not viable and exhibit two distinct phenotypes. They either have a severe defect in placental development in which the allantois fails to fuse with the chorion, or they display several abnormalities in the developing heart, including a reduction of the compact layer of the ventricular myocardium and intraventricular septum (Kwee et al. 1995). In fetal intrauterine growth restriction, placental expression of VCAM1 is decreased compared with normal placentas (Rajashekhar et al. 2003). However, in pre-eclamptic placentas the production of VCAM1 and other adhesion molecules has been shown to be similar to that in normotensive pregnancies (Jaakkola et al. 2000, Tziotis et al. 2002). PECAMs help macrophages to recognize aged neutrophils, and during cell digestion, macrophages release more TFG-β, which promotes cell proliferation and stimulates tissue repair.

Glycodelin-A

Endometrial glycodelin-A (GdA, PP14) is a progesterone- and relaxin-regulated glycoprotein secreted into the uterine cavity by secretory and decidualized endometrial glands (Julkunen et al. 1986a, 1986b, Fay et al. 1990). GdA has contraceptive and immunosuppressive properties. Because of its inhibitory activity on sperm–egg binding, GdA is absent during ovulation and its production increases in the late-secretory phase of the menstrual cycle. It is abundant in decidualized endometrium during blastocyst implantation and in the first trimester of pregnancy. GdA has been found to inhibit NK cell activity and abrogate the enhancement of IL-2-induced cytotoxicity. In addition, there is evidence that GdA increases the production of IL-6, an essential factor for successful implantation, from the secretory endometrium, but not from the proliferative endometrium (Laird et al. 2000).
These findings, along with the high GdA concentration at the feto-maternal interface, suggest that GdA plays an important role in the feto-maternal defence mechanism and endometrial receptivity (Clark et al. 1996, Seppala et al. 2002). A recent study by Lam et al. (Lam et al. 2009) showed that GdA inhibits extravillous trophoblast invasion mainly by suppressing the activity of MMP2 and MMP9. Decreases in maternal serum glycodelin concentrations are associated with early spontaneous and recurrent miscarriage (Seppala et al. 2002).

2.2.4 Extracellular matrix degradation

The extracellular matrix (ECM) is composed of a variety of proteins and polysaccharides assembled into an organized network that provides structural support for cells. In addition, the ECM separates different tissue types, regulates intercellular communication, and sequestrates and deposits a wide range of cellular growth factors. Remodelling of the ECM through controlled proteolysis and re-synthesis of ECM components is a crucial part of normal tissue growth and differentiation.

Proteinases

For successful invasion into the decidua, trophoblast cells must recognize the different components of the interstitial matrix and basement membrane in order to break them down (Manyonda & Choy 1999). To control and facilitate this invasion, vascular permeability of the endometrium increases, which leads to oedema of stromal tissue. Secondly, endometrial stroma modifies the composition of the ECM by secreting several proteinases, activators and their inhibitors which are involved in digestion of the ECM. Matrix metalloproteinases (MMPs) proteolyse components of ECM and are closely associated with invading trophoblasts (Shimonovitz et al. 1994). MMPs are regulated by specific tissue inhibitors, TIMPs (tissue inhibitor of MMPs) and are activated by plasmin. Thus, levels of MMPs rise in association with plasminogen activators (PAs), urokinase PA (uPA) and tissue-type PA, which are members of the family of serine proteinases. The trophoblast cells secrete many MMPs and uPA during the first trimester. Up-regulation of MMP-9 (gelatinase B) has been associated with superficial trophoblast invasion and an increased risk of pre-eclampsia. Invading trophoblasts are also able to secrete gonadotrophin-releasing hormone (GnRH), which seems to diminish the activity of TIMPs in the endometrial stroma (Raga et al. 1999).
Integrins

The adhesion of extravillous trophoblasts to different ECM components requires the interaction of integrins and cadherins, the cell membrane receptors that bind to the trophoblasts. Integrins are heterodimeric glycoproteins with two subunits: α and β. Their different combinations form several integrin molecules that bind to various components of ECM ligands including laminin, fibronectin and collagen. Integrin α5β3 is expressed at the implantation site in human endometrium at days 20–24 of the menstrual cycle and may be necessary for embryonic attachment (Lessey et al. 1995). Proliferative, non-invasive cytotrophoblasts produce integrin α6β4 (a laminin receptor which is a component of basement membrane), α6β6 and α5β1 (fibronectin receptor), which restrain invasion. Zhou et al. (Zhou et al. 1993a, 1993b, 1997) demonstrated dysfunctional placental expression of several adhesion molecules, i.e. failure of down-regulation of molecules present in the non-invasive phenotype, and failure of up-regulation of molecules present in the invasive cytotrophoblast, α5β1 (collagen I and IV and laminin receptor), α5β3 and α5β5. This failed integrin switch may be responsible for the pathological findings of shallow trophoblast implantation with limited vascular invasion. In addition, during normal placentation, extravillous trophoblasts down-regulate the expression of cadherin E (epithelial) and up-regulate the expression of cadherin VE (vascular endothelial) to promote trophoblast invasion (Damsky et al. 1992, Zhou et al. 1993b). Cadherins are molecules that mediate cell-cell adhesion.

Fibronectin

The insoluble form of fibronectin is an important component of the ECM. It is a glycoprotein which binds to other ECM components, such as integrins, collagen, fibrin and heparan sulphate. Fibronectin maintains cell shape by organizing the intracellular cytoskeleton, and it helps to stabilize attachment of the ECM to cells by acting as a binding site for cell surface receptors. Dallas et al. (Dallas et al. 2005) showed that fibronectin regulates TGF-β availability. In addition, fibronectin facilitates the fusion of cytotrophoblasts into giant cells (Dallas et al. 2005). The observation that interstitial trophoblastic cells in the placental bed are surrounded by thick deposits of fibronectin suggests that fibronectin secretion by trophoblasts is an autoregulatory mechanism to control the depth of invasion (Pijnenborg et al. 1996b).
2.2.5 Placental proteins

*Human chorionic gonadotrophin*

Human chorionic gonadotrophin (hCG) is one of the earliest proteins produced by the embryo. Its mRNA is transcribed as early as at the 8-cell stage (Jurisicova *et al.* 1999) and blastocysts produce hCG before implantation (Bonduelle *et al.* 1988). The peak of placental hCG production is reached between 10–11 weeks of gestation; thereafter its secretion decreases. HCG prevents luteolysis and stimulates progesterone production (Keay *et al.* 2004). Recent studies have shown that hCG plays an important role in immune tolerance of the fetal allograft and in regulation of decidual angiogenesis. During implantation and early pregnancy, hCG increases the numbers of uNK cells (Kane *et al.* 2009) and peripheral regulatory T cells (Zenclussen 2006b), and increases C3 and C4 gene expression in the baboon endometrium (Sherwin *et al.* 2007). In addition, hCG down-regulates Th1 cells and up-regulates Th2 cells, thus promoting the production of Th2-derived cytokines. Leukaemia inhibitory factor (LIF), VEGF and MMP-9 are significantly activated by hCG (Licht *et al.* 1998), whereas IGFBP-1 is inhibited by it (Tsampalas *et al.* 2010). Dong *et al.* (Dong *et al.* 2008) showed in a recent large proteomic study that hCG inhibits T lymphocytes.

Toth *et al.* (Toth *et al.* 2001) have shown that hCG reduces vascular resistance in human uterus and decreases levels of vasoconstrictive prostanoids of the vascular wall. HCG promotes angiogenesis by supporting the migration of uterine endothelial cells and the formation of capillary structures (Zygmunt *et al.* 2002).

*Pregnancy-associated plasma protein A*

Pregnancy-associated plasma protein A (PAPP-A, pappalysin) is a metalloproteinase which is produced mainly by trophoblast cells. It is present in the maternal circulation soon after implantation, with increasing concentrations during pregnancy (Westergaard *et al.* 1983, Lawrence *et al.* 1999). Maternal PAPP-A levels are higher than normal in multiple and primigravid pregnancies. There is also a positive correlation between PAPP-A levels and placental weight (Westergaard *et al.* 1983). PAPP-A cleaves IGFBP-4 and -5, thus potentiating the action of insulin-like growth factors (Lawrence *et al.* 1999, Laursen *et al.* 2001). Because of its proteolytic activity, PAPP-A acts as a regulating and modulating protein of the insulin-like growth factor system. This is critical for placental formation and
regulation of fetal growth. Low maternal serum PAPP-A levels are associated with chromosomal abnormalities and many pregnancy complications such as early pregnancy failure, pre-eclampsia and intrauterine fetal demise (Westergaard et al. 1983, Ong et al. 2000). Absence of PAPP-A results in fetal deformities (Conover et al. 2004), and IGFBP-4 deficient mice are smaller than their wild-type littermates (Ning et al. 2008).

Pregnancy-specific β 1 glycoprotein 1

Trophoblastic cells produce pregnancy-specific β 1 glycoprotein 1 (PSG1), which is a member of the carcinoembryonic antigen family (Bohn 1971). In vitro-cultured human pre-embryos from women participating in in vitro fertilization programmes start to release PSG1 on day 3 or 4 after fertilization, coinciding with attachment of the blastocyst into the uterine wall (Dimitriadou et al. 1992). PSG1 affects many different cell types in early placenta. In endothelial and extravillous trophoblast cells, PSG1 induces production of the pro-angiogenic factors TGF-β and VEGF (Snyder et al. 2001, Ha et al. 2010). In the immune system, PSG1 activates monocytes to synthesize anti-inflammatory cytokines and promotes alternative macrophage activation – a shift from Th1- to Th2-mediated immunological responses (Motran et al. 2003). Low PSG1 levels in maternal serum are associated with pregnancy complications such as spontaneous abortion, pre-eclampsia and IUGR (Bersinger & Odegard 2004, Bersinger et al. 2004, Pihl et al. 2009).

Chorionic somatomammoprotein 1

Chorionic somatomammoprotein 1 (CHS1, human placental lactogen (HPL)) structurally and functionally resembles pituitary growth hormone (GH) and prolactin (PRL). GH and PRL are pro-angiogenic hormones that regulate growth, energy metabolism, immune responses and capillary formation (Ben-Jonathan et al. 2008, Clapp et al. 2008). PRL promotes angiogenesis in an autocrine manner and also indirectly by inducing the synthesis of VEGF and fibroblast growth factor in decidual and immune cells (Sravastava et al. 1998, Goldhar et al. 2005). CSH1 signals via the PRL receptor and may be an important contributor to increased blood vessel growth during pregnancy (Clapp et al. 2009).
Placental protein 13

The role of placental protein 13 (PP13) in normal pregnancy is not yet fully understood. It seems to have special haemostatic and immunological functions at the feto-maternal interface (Than et al. 2004). Serum levels of PP13 increase during normal pregnancy. Low levels of PP13 have been detected in first trimester serum samples from women subsequently developing IUGR or early onset pre-eclampsia (Chafetz et al. 2007, Spencer et al. 2007, Huppertz et al. 2008).

2.2.6 Interleukins

The traditional role of cytokines is perceived in their immunoregulatory properties. However, they also promote mitogenesis and apoptosis in non-immune cells (Kawamura et al. 2007). Cytokines have unique properties including pleiotropism (each cytokine has multiple target cells in an array of different organs) and their responses may differ depending on cell type. Interleukin 1 (IL-1), IL-11 and LIF are unequivocally important cytokines as regards blastocyst attachment to the uterine wall and implantation.

IL-1

Interleukin 1 is the most investigated cytokine in the human reproductive system, and there are two agonists, IL-1α and IL-1β. They are involved in the implantation process and blastocyst development prior to implantation (Krussel et al. 2003). In mice, increasing levels of IL-1α and IL-1β have been detected in endothelial cells from day 3 onwards, with maximal levels between days 4 and 5 (Krussel et al. 1998) when blastocyst implantation occurs. In addition, intraperitoneal administration of IL-1 receptor antagonist (IL-1Ra) from day 3 to day 6 of pregnancy in mice inhibits implantation (Simon et al. 1998). The mechanism by which IL-1Ra interferes with embryo implantation is most likely related to alterations in adhesion molecules, especially αvβ3 integrin (ECM receptor for vitronectin, fibronectin and osteopontin) (Lessey et al. 1995). In vitro-fertilized, cultured human embryos produce both IL-1α and IL-1β, and their high concentrations in culture media have been associated with successful implantation after intrauterine embryo transfer (Sheth et al. 1991). However, not all investigators have been able to confirm these results (Seifer et al. 1993). Huang et al. (Huang et al. 1998) demonstrated that IL-1 regulates MMPs and TIMPs and thus has an important role in blastocyst attachment and implantation via ECM degradation.
**LIF and IL-11**

Leukaemia inhibitory factor and IL-11 are members of the IL-6 family. LIF-deficient (LIF-/-) female mice lack decidualization and are unable to support implantation, irrespective of the blastocyst genotype, whereas LIF-/- blastocysts can implant in wild-type females. Furthermore, mice with a null mutation in the IL-11 receptor (IL-11R) gene are infertile as a result of insufficient decidualization (Robb et al. 1998). The main mechanisms by which LIF and IL-11 are involved in implantation concern regulation of integrin, TIMP (Marwood et al. 2009) and HLA-G expression (Bamberger et al. 2000).

High LIF expression is an indicator of receptive endometrium in fertile women and ovarian stimulation has been shown to reduce LIF expression in comparison with that in natural cycles (Chen et al. 2008). However, in infertile women the data on endometrial LIF expression and secretion is controversial. Serafini et al. (Serafini et al. 2009) showed that LIF immunoexpression intensity was strongly associated with successful pregnancy after IVF, whereas Makker et al. (Makker et al. 2009) found no difference in endometrial LIF immunoexpression between fertile and infertile women. In LIF-/- mice, implantation can be rescued by LIF injections (Stewart et al. 1992, Chen et al. 2000). However, recombinant human LIF injections did not improve the outcome of IVF in women with recurrent implantation failure (Brinsden et al. 2009). In mice, low-dose aspirin treatment during the implantation window increased LIF and integrin β3 expression (Zhao et al. 2010). The result may indicate increased endometrial receptivity in connection with low-dose aspirin treatment.

### 2.2.7 Prostanoids and cyclo-oxygenases

Prostanoids are fatty acids that are synthesized from arachidonic acid, a component of cell membranes. Prostanoid synthesis begins when arachidonic acid is released from membrane phospholipids by the enzyme phospholipase A2. Arachidonic acid is then converted to prostaglandin (PG) H2 by the enzyme cyclo-oxygenase (COX) and further to primary PGs, prostacyclin and thromboxane A2 via their own synthases (Figure 3).

Prostacyclin is an endothelial cell product that has vasodilatory properties and the ability to inhibit platelet aggregation. In the placenta, prostacyclin primarily originates from placental vascular tissue, but trophoblast cells are capable of producing small amounts of prostacyclin (Nelson & Walsh 1989, Walsh & Wang...
During normal pregnancy, the production of prostacyclin is increased when compared with the non-pregnant state, as evidenced by elevated maternal plasma concentrations of its stable metabolite, 6-keto PGF$_{1\alpha}$, and elevated levels of its urinary metabolites (Lewis et al. 1980, Goodman et al. 1982). Placental prostacyclin is present early in gestation and increases sharply between 6 and 12 weeks of pregnancy (Rakoczi et al. 1985). Prostacyclin modulates the actions of some hormones and, in particular, diminishes the sensitivity of maternal vessels to angiotensin II, which is a vasoconstrictive agent (Merviel et al. 2004). In contrast to prostacyclin, thromboxane A$_2$ is a powerful vasoconstrictor that induces platelet aggregation and uterine contractility. In the placenta, trophoblasts are the major source of thromboxane A$_2$. During early normal rat pregnancy, the intravascular plugs formed by the extravillous trophoblast cells at the junction between spiral arteries and the intervillous space inhibit membrane lipid peroxidation and diminish thromboxane A$_2$ levels (Davidge et al. 1994). Accordingly, the increase in the prostacyclin/thromboxane ratio promotes vasodilatation and decreases vascular resistance. HCG regulates the placental vascular bed by increasing prostacyclin synthesis and decreasing the synthesis of vasoconstrictive agents such as prostaglandin F$_{2\alpha}$ and thromboxane A$_2$.

Cyclo-oxygenase exists in two isoforms, COX-1 and COX-2. The constitutive form, COX-1, supports physiological functions, whereas the inducible form, COX-2, becomes up-regulated by inflammatory mediators (Vane & Botting 2003) at sites of cell damage and inflammation. There is a unique pattern of expression of COX-1 and COX-2 genes during the peri-implantation period in the mouse uterus (Chakraborty et al. 1996, Lim et al. 1997a). COX-1 is expressed in endometrial epithelial cells during fertilization, but its expression becomes undetectable by the time of implantation. In contrast, COX-2 is expressed in endometrial epithelial and stromal cells solely at the site of blastocyst implantation. COX-2-deficient mice have been shown to have complete implantation failure (Lim et al. 1997a), indicating that COX-2 expression and prostanoids are critical in this process. Although prostaglandins appear to be essential for implantation, Norwitz & Wilson (Norwitz & Wilson 2000) showed that the administration of exogenous prostaglandins (intravenously, intra-amniotically or vaginally) induces abortion in mice at any stage of gestation.
<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect on placentation</th>
<th>Receptor</th>
<th>Activator</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>Low oxygen tension before 10 weeks’ gestation protects developing embryo from teratogenic effect of oxygen free radicals, and maintains stem cells in a fully pluripotent state</td>
<td>Low $O_2$ tension</td>
<td>High $O_2$ tension</td>
<td></td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Promotes cytotrophoblastic proliferation and villous vasculogenesis</td>
<td>Low $O_2$ tension</td>
<td>Hypoxia</td>
<td>sflt-1</td>
</tr>
<tr>
<td>VEGF1 and VEGF2</td>
<td>Promotes vasculo- and angiogenesis</td>
<td>VEGFR-1 (flt-1), VEGFR-2 (KDR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang-1</td>
<td>Promotes endothelial maturation and vascular stabilisation</td>
<td>Tie-2</td>
<td></td>
<td>Ang-2</td>
</tr>
<tr>
<td>Ang-2</td>
<td>Promotes angiogenesis in the presence of VEGF</td>
<td>Tie-2</td>
<td></td>
<td>Ang-1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Promotes trophoblast proliferation</td>
<td>Endoglin</td>
<td></td>
<td>Vasoerin</td>
</tr>
<tr>
<td>Activin</td>
<td>Promotes decidualization and implantation, enhances cytotrophoblast column formation and differentiation to invasive phenotype, promotes cytotrophoblast fusion and syncytialization</td>
<td>Activin type 1 and 2 receptors</td>
<td></td>
<td>Follistatin, inhibin, activin-binding protein</td>
</tr>
<tr>
<td>Inhibin</td>
<td>Inhibits steroidogenesis and hCG production from trophoblasts</td>
<td></td>
<td></td>
<td>Activin</td>
</tr>
<tr>
<td>IGF I and IGF II</td>
<td>Promotes fetal weight gain and placental growth, enhances cytotrophoblast proliferation and inhibits apoptosis, promotes trophoblast invasion (IGF II)</td>
<td>IGF1R</td>
<td>IGFBPs</td>
<td>IGFBPs</td>
</tr>
<tr>
<td>IGFBP 1–6</td>
<td>Regulates IGF I and IGF II availability by binding to these molecules</td>
<td></td>
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<tr>
<td>uNKs</td>
<td>Mediate decidual reaction, function both as activators and inhibitors in trophoblast invasion and angiogenesis depending on cytokines produced</td>
<td></td>
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<tr>
<td>HLA-G</td>
<td>Protects trophoblasts from uNK cell attack</td>
<td>KiRs</td>
<td></td>
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<tr>
<td>VCAM</td>
<td>Promotes trophoblast migration and invasion</td>
<td>Tie-1</td>
<td>Cytokines</td>
<td>Integrin α4</td>
</tr>
<tr>
<td>Glycodeolin A</td>
<td>Decreases feto-maternal defence mechanisms, inhibits trophoblast invasion by suppressing the activity of MMP2 and MMP9</td>
<td></td>
<td>Progesterone, relaxin</td>
<td></td>
</tr>
<tr>
<td>MMPs</td>
<td>Promote trophoblast invasion by proteolysing ECM components</td>
<td></td>
<td>Plasmin</td>
<td>TIMPs</td>
</tr>
</tbody>
</table>

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### 2.3 Placenta-related pregnancy complications

#### 2.3.1 Pre-eclampsia and pregnancy-induced hypertension

Pre-eclampsia and pregnancy-induced hypertension (PIH) are pregnancy-specific disorders characterized by increased blood pressure after 20 weeks of gestation in previously normotensive women. In pre-eclampsia, another diagnostic criterion is
proteinuria (Roberts et al. 2003, Furuya et al. 2008). In severe cases, pre-eclampsia may lead to life-threatening seizures (eclamptic episodes). Hypertensive pregnancy disorders affect 10–12% of all pregnancies (Duley 2008). Worldwide, they are the leading cause of maternal and fetal morbidity and mortality (Schutte et al. 2008). The risk factors of PIH and pre-eclampsia are heterogeneous, including primiparity, previous pre-eclampsia, antiphospholipid syndrome, chronic hypertension, renal disease, multiple pregnancy, thrombophilias, obesity, maternal age > 40 years and IVF. So far, the only effective therapy for pre-eclampsia is removal of the placenta.

The underlying aetiology of hypertensive pregnancy complications remains elusive, but it is generally accepted that abnormal early trophoblast invasion is a key step in the development of pre-eclampsia (Kaufmann et al. 2003). In pre-eclampsia, the extent of interstitial trophoblast invasion in the placenta is rudimentary and superficial (Brosens et al. 1972, Roberts et al. 1989, Lim et al. 1997b) and maternal vessels containing cytotrophoblasts are scarce (Zhou et al. 1997). This abnormal trophoblast cell invasion into maternal decidua, myometrium and spiral arteries is thought to be the initial event of pre-eclampsia (Lim et al. 1997b, Granger et al. 2001). In pre-eclampsia, the spiral arteries remain narrow and maintain their vasoconstrictive properties (Meekins et al. 1994). This can lead to reduced uteroplacental circulation, resulting in relative placental bed ischaemia and oxidative stress, which may cause widespread activation and dysfunction of the maternal vascular endothelium and finally clinical manifestations of pre-eclampsia (Bersinger et al. 2003, Redman & Sargent 2005).

Placentas from women with pre-eclampsia express lower levels of MMP-9, HLA-G, CSH1 and α1β1-integrin than those from women with normal pregnancies (Zhou et al. 1997, Merviel et al. 2001, 2004). Moreover, the switch from E-cadherin to VE-cadherin does not occur, and VCAM and PECAM are not produced sufficiently (Zhou et al. 1993b, Zhou et al. 1997). These results suggest that in pre-eclamptic placenta undifferentiated cytotrophoblasts fail to adopt the invasive vascular adhesion phenotype of endothelial cells (Zhou et al. 1997, Pijnenborg et al. 1998a, 1998b). Interestingly, levels of PAPP-A have been reported to be decreased in women who develop placenta-related pregnancy complications later in pregnancy (Smith et al. 2002, Bersinger & Odegard 2004). Similar findings have been demonstrated concerning PP13. Accumulating evidence exists of alterations of expression of vascular growth factors and their receptors in women destined to develop pre-eclampsia later in pregnancy. Serum concentrations of sflt-1 are elevated prior to onset of clinical symptoms of pre-eclampsia (Levine et al. 2004), as early as in the first trimester (Baumann et al. 2007). Accordingly, levels of circulating free
VEGF and PlGF are decreased (Clark et al. 1998, Zhou et al. 2002). However, a study based on maternal serum proteome analysis revealed similar angiogenic and anti-angiogenic protein levels in normotensive women and in women with later pre-eclampsia when samples were collected at 8–14 weeks of gestation (Rasanen et al. 2010). Salomon et al. (Salomon et al. 2003) and Zwahlen et al. (Zwahlen et al. 2007) observed elevated serum levels of inhibin A and activin A (members of the TGF-β family) as early as in the first trimester in women who subsequently developed pre-eclampsia, compared with controls. An association between elevated second trimester hCG levels and an increased risk of pre-eclampsia has been confirmed by many investigators (Muller & Bussieres 1996, Audibert et al. 2005).

In vitro studies have demonstrated that trophoblasts from pre-eclamptic placentas produce more thromboxane \( A_2 \) than trophoblasts from normal placentas (Makila et al. 1984, Walsh 1989, Walsh & Wang 1995). The imbalance of increased thromboxane \( A_2 \) production and reduced prostacyclin production by pre-eclamptic trophoblasts is believed to contribute to the increased vasoconstriction in pre-eclampsia. In addition, placental hypoxia further increases thromboxane \( A_2 \) production in both normal and pre-eclampsia placentas (Bowen et al. 2005). Placental vessels lack autonomic innervation and therefore local hormones and vasoactive substances produced by the placenta, such as prostanoids, play an important role in regulation of feto-placental vascular contractility. While many investigators have reported lower prostacyclin metabolite levels (Ylikorkala et al. 1986, Minuz et al. 1987, Kaaja et al. 1995, Liu et al. 1998) and higher thromboxane \( A_2 \) metabolite levels (Minuz et al. 1988, Fitzgerald et al. 1990) in urine or plasma of hypertensive and pre-eclamptic women compared with normotensive women, some studies failed to confirm these results (Smith et al. 1995, Paarlberg et al. 1998, Mills et al. 1999). Contradictory findings may be explained by the fact that plasma levels of thromboxane \( A_2 \) primarily reflect the production of thromboxane by platelets in the circulation. Furthermore, maternal circulating levels of thromboxane \( A_2 \) are not increased in mild pre-eclampsia, but they are increased significantly in severe forms of the disease (Wang et al. 1991). This is consistent with platelet activation in severe pre-eclampsia, while prostacyclin levels are decreased significantly in both mild and severe pre-eclampsia.

### 2.3.2 Intrauterine growth restriction

Fetal growth is regulated by genomic and environmental mechanisms. Of somatotrophic factors, the IGF system plays the most important role. However,
development of the maternal and feto-placental vascular system is critical for normal embryonic and fetal growth. About 30% of women with pre-eclampsia have a growth-restricted fetus (Walker 2000), but intrauterine fetal growth restriction (IUGR) can be an independent finding without maternal symptoms of pre-eclampsia. Abnormal development of the placental circulatory system, similar to that in pre-eclamptic placentas, has been shown to be present in cases of IUGR. However, severe IUGR is specifically associated with failure to elaborate a normal peripheral villous tree in the placenta, and there are high levels of apoptosis in villous trophoblasts (Kingdom et al. 2000). This leads to a decreased oxygen level in the intervillous space and abnormal nutrient exchange between mother and fetus. Villous formation is dependent on successful angiogenesis and thus VEGF, PlGF and their receptors play a crucial role in villous development. Abnormal development of the villous tree results in placental blood flow abnormalities that can be observed in Doppler ultrasonographic examination (Giles et al. 1985).

2.4 IVF and ICSI treatment

*In vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are treatment options for couples suffering from infertility. The clinical pregnancy rate among women undergoing fresh embryo transfer (ET) after IVF or ICSI varies from 27 to 34% per transfer (Nybøe Andersen et al. 2009, de Mouzon et al. 2010). The most important factors that affect the outcome of IVF and ICSI treatments are age of the woman, body mass index, number and quality of oocytes and embryos, number of embryos transferred, success of embryo transfer and endometrial receptiveness (Weckstein et al. 1997, Ebner et al. 2000, Terriou et al. 2001, Tomas et al. 2002). However, when top-quality embryos are available, a high pregnancy rate can be achieved by elective single ET in selected women (Martikainen et al. 2001, Tiitinen et al. 2003, McLernon et al. 2010).

2.4.1 Assessment of outcome of IVF and ICSI

*Ovarian responsiveness*

A poor response to ovarian stimulation leads to a decreased pregnancy rate after IVF and ICSI. It occurs in 9–24% of women undergoing ovarian stimulation (Keay et al. 1997, Sunkara et al. 2007). Abnormal circulating levels of estradiol and FSH during stimulation, poor growth of follicles and a low level of retrieved oocytes (Broekmans
et al. 2006), as well as poor embryo quality (Fisch et al. 2008), indicate poor ovarian response. Ovarian responsiveness to gonadotrophins deteriorates with increasing age (Beckers et al. 2002), though a poor response can occur at any age (Check et al. 1998b).

A poor ovarian response is associated with an extended treatment period and a need for high doses of gonadotrophins. Various strategies, such as modifying the stimulation protocol, or the use of adjuvants have been implemented to improve ovarian responsiveness, but the results have been contradictory (Keay et al. 1997, Tarlatzis et al. 2003, Lok et al. 2004).

**Implantation and clinical pregnancy rate**

Traditionally, implantation rate and clinical pregnancy rate are used as parameters to evaluate the success of IVF and ICSI treatments. However, implantation and clinical pregnancy rates include cases of pregnancy failure and they are always higher than the live-birth rate, which is the most objective measure of outcome in IVF. Most investigators, however, have used implantation rate or clinical pregnancy rate as a main outcome variable (Rubinstein et al. 1999, Urman et al. 2000, Stern et al. 2003, Moini et al. 2007, Dirckx et al. 2009).

**2.4.2 Factors affecting outcome of IVF and ICSI**

The two most important factors affecting embryo quality and endometrial receptivity are the number of good-quality embryos transferred and the woman’s age.

**Number of oocytes and quality of embryos**

Ovarian stimulation is a key component of assisted reproductive treatment. The increase in the number of oocytes retrieved enables the selection of the best-quality embryos for transfer (Baart et al. 2009). A low number of oocytes is associated with a poor clinical outcome and is believed to represent ovarian ageing (Beckers et al. 2002, Tarlatzis et al. 2003). Despite the lack of consensus regarding the exact definition of poor response (Klinkert et al. 2004), the relationship between the number of oocytes retrieved and success rates is well documented (Keay et al. 1997).

Several studies have shown a relationship between oocyte and embryo quality (Xia 1997, Loutradis et al. 1999, Ebner et al. 2000). The morphological aspects that are related to embryo quality include cleavage rate and speed, degree of fragmentation, appearance of the cytoplasm and blastomere irregularity (Van
Royen et al. 1999, Ziebe et al. 2001). Together with mitotic division and grading score assessment, these parameters are used to determine the number of embryos to be transferred, or frozen to avoid multiple pregnancies, without affecting the success rate of IVF and ICSI treatment. The definition of a top-quality embryo includes four regular cells, no multinucleated blastomeres, $\leq 20\%$ fragmentation and four or five blastomeres on day 2, or seven or more blastomeres on day 3 (Van Royen et al. 1999). Saldeen and Sundström (Saldeen & Sundstrom 2005) reported that the transfer of single top-quality embryo resulted in a pregnancy rate of 44%, while after the transfer of one non-top-quality embryo the pregnancy rate was 19%.

**Uterine receptivity**

It has been estimated that uterine receptivity accounts for 31–64% of cases of successful implantation (Rogers et al. 1986). The human endometrium is receptive to the blastocyst only during a very short time period in the luteal phase, (period of maximum uterine receptivity for implantation) (Duc-Goiran et al. 1999).

A favourable endometrial milieu for ET has been predicted ultrasonographically by measuring blood flow velocities and blood flow velocity waveform indices in the uterine, subendometrial and endometrial arteries (Zaidi et al. 1996, Schild et al. 2001, Chien et al. 2002, Isaksson et al. 2003, Puerto et al. 2003, Merce et al. 2008) and by assessing endometrial thickness, morphology (Zaidi et al. 1995, Schild et al. 2001) and volume (Yaman et al. 2000, Schild et al. 2001). None of these methods have proved to be useful. Endometrial thickness is thought to be an important factor for successful IVF and ICSI treatments. However, variation in endometrial thickness overlaps among women with and without successful IVF. There is a consensus of opinion that endometrial thickness over 6 mm at the time of ET is adequate for normal conception (Friedler et al. 1996), but successful pregnancy is possible even in cycles with a maximal endometrial thickness of only 4 mm (Sundstrom 1998, Check et al. 2003).

### 2.4.3 Special features of placentation after IVF and ICSI

than in spontaneously conceived normal singleton pregnancies. Furthermore, in IVF and ICSI pregnancies, increased concentrations of maternal serum inhibin-A (Tul & Novak-Antolic 2006) and decreased levels of PSG1 and CSH1 (Bersinger et al. 2004) have been shown during the first trimester compared with naturally conceived pregnancies. Second trimester maternal serum β-hCG levels are elevated in unstimulated frozen-thawed embryo cycles, suggesting that ovarian hyperstimulation and the IVF procedure itself are not the primary causes of alterations in the levels of biomarkers (Perheentupa et al. 2002). A recent study by Ranta et al. (Ranta et al. 2010) demonstrated that subfertile women whose time-to-pregnancy interval was over two years, had maternal serum protein levels similar to those described in IVF pregnancies. These results indicate that biomarker alterations could be related to subfertility and infertility rather than to use of ovarian hyperstimulation techniques.

2.4.4 Special features of pregnancies after IVF and ICSI

Infertility itself can increase the risk of adverse obstetric and perinatal outcomes. Joffe and Li (Joffe & Li 1994) showed an increased risk of miscarriage among subfertile spontaneously conceiving women compared with women with normal fertility. Ranta et al. (Ranta et al. 2010) suggested that there may be a negative correlation between birth weight and time-to-pregnancy interval in spontaneously conceiving women. In addition, the incidence of pre-eclampsia tended to be increased in women whose time-to-pregnancy interval was over two years (Ranta et al. 2010). Draper et al. (Draper et al. 1999) reported a 3-fold increase in perinatal mortality in subfertile women.

In IVF and ICSI pregnancies, the incidence of low birth weight, preterm delivery, hypertensive pregnancy complications, IUGR, placenta praevia, Caesarean sections, stillbirth, neonatal death, as well as cerebral palsy, are increased compared with spontaneous pregnancies (Koivurova et al. 2002, Jackson et al. 2004, Klemetti et al. 2006, Schieve et al. 2007, Sazonova et al. 2011). In IVF patients the risk of developing PIH is about 2-fold greater (Allen et al. 2006) and the risk of pre-eclampsia is about 2.7-fold greater (Shevell et al. 2005) compared with spontaneous pregnancies, even after adjusting for maternal age, parity and multiple gestations. In a meta-analysis carried out by Jackson et al. (Jackson et al. 2004), the incidence of IUGR was 2.7-fold and that of perinatal mortality 2.2-fold higher in singleton IVF pregnancies when compared with spontaneous pregnancies.
2.5 Proteomic analysis in reproductive medicine

2.5.1 Methodology

Proteomic analysis involves the systematic separation, identification and characterization of proteins present in a biological sample. Analysis requires resolution of the total protein content followed by identification and quantification of the separated proteins. Proteome separation is commonly accomplished by two-dimensional gel electrophoresis (2DE), a technique able to separate several thousand proteins in one analysis (O’Farrell 1975, Klose & Kobalz 1995), followed by identification of the separated proteins by mass spectrometry (MS).

In 2DE (Figure 2a) the first step is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pIs). The second step is SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weight (Figure 2). Several requirements must be met to allow the extraction of all protein components in a system, including hydrophobic membrane proteins, proteins with extreme pI values (below pH 3 and above pH 10), as well as proteins with low concentrations in the presence of other abundant proteins, and there must be high-resolution separation of the spots. Several improvements have been made to the original two-dimensional gel electrophoresis procedure to meet these requirements (Gorg et al. 1997, Wildgruber et al. 2000, Zhang et al. 2001).
Fig 2. a) A schematic diagram of differential 2D gel electrophoresis. Samples from a study group are each labelled with a different fluorescent dye tag and then mixed and resolved on the same gel, separated by isoelectric focusing and by molecular weight. The gel is then scanned at different wavelengths and scanned images are overlayed to display differential protein expression.
Mass spectrometry (Figure 2b) is a technique used to measure the mass-to-charge ratio of charged particles and it is used for the identification of proteins separated by 2DE (Aebersold 2003, Aebersold & Mann 2003). A mass spectrometer consists of three basic parts, 1) an ion source, 2) a mass analyser, and 3) a detector system. The most commonly used ion source systems in MS are matrix-assisted laser desorption (MALDI), and electrospray (ESI) systems. MALDI sublimates and ionizes the samples out of a dry crystalline matrix via laser pulses, whereas ESI ionizes analytes out of solution and is normally coupled with liquid-chromatography (LC). The mass analysers used in MS techniques are time-of-flight (TOF), ion trap, quadrupole and Fourier transform ion cyclotron analysers. A MALDI ion source is usually coupled to a TOF analyser and ESI to ion trap or quadrupole instruments (Aebersold & Mann 2003). The final element of a mass spectrometer, the detector, records the charge induced or current produced when an ion passes...
by or hits a surface. Protein/peptide identification can be conducted by comparing a small number of enzymatically digested peptide masses to peptide mass maps in a database (e.g. Prowl and ProteinProspector), or by single peptide (amino acid) tag-analysis in multiple MS procedures performed in tandem (tandem mass spectra or MS/MS), which is usually employed for added specificity. Peptide sequence databases for searching mass spectra include, for example, OpenSea, TurboSequest and X!Tandem. In theory both MALDI and ESI are suitable for protein identification by peptide mass fingerprinting and by sequence analysis. In practice, however, only ESI is used in sequencing.

2.5.2 Proteomic studies in early pregnancy

Genomic and proteomic studies have focused on signalling pathways associated with blastocyst formation and uterine receptivity to blastocyst implantation (Natale et al. 2004, Daikoku et al. 2005). The vast amount of proteins derived from decidua, placenta, blastocyst and ECM have been implicated in the cell-to-cell and cell-to-ECM interactions essential for successful implantation and pregnancy (Lala & Hamilton 1996, Aplin 1997, Kimber 2000, Lindhard et al. 2002).

The proteome of luteal phase compared with non-receptive endometrium has been studied to gain insights into endometrial receptivity for blastocyst implantation. Dominiguez et al. (Dominguez et al. 2009) reported 32 differentially expressed proteins in luteal phase endometrium, although only annexin A2 (a protein associated with proliferation and fibrinolysis) and stathmin I (which promotes proliferation by increasing mitotic activity) were consistently up- or down-regulated. In particular, they were up-regulated seven days after the luteal hormone (LH) surge, which is considered to be the time of implantation.

Proteomics has been used to analyse the proteome of human blastocysts. Katz-Jaffé et al. (Katz-Jaffé et al. 2006) used surface-enhanced laser desorption/ionization (SELDI, a variant of MALDI-TOF-MS to identify biomarkers connected to the development and viability of the human embryo. They observed several differentially expressed proteins in early and expanded blastocysts, as well as in developing blastocysts and degenerating embryo. The candidate proteins include transcription factors mediating the Wnt signalling pathway (a network of proteins required for basic developmental processes) and an apoptotic protease-activating factor. The results suggest that protein expression profiles can be related to morphology during embryo development and that in degenerating embryos apoptotic and growth-inhibiting pathways are activated.
2.5.3 Proteomics and hypertensive pregnancy complications

Mine et al. (Mine et al. 2007) were the first to publish a comprehensive proteome analysis of human placental tissue (both normal and pre-eclamptic placentas) using MALDI-TOF-MS and LC-MS/M. About 60% of the total protein spots on the proteome map were pregnancy-related proteins. In pre-eclamptic tissues, placental dynactin (a protein related to cell turnover) was significantly over-expressed compared with normal placentas. Proteomic analyses of placental and blood samples have revealed several differentially expressed proteins in pre-eclampsia in comparison with tissues from normotensive women (Myers et al. 2004, Jin et al. 2008, Gharesi-Fard et al. 2010). A recent study by Gharesi-Fard et al. (Gharesi-Fard et al. 2010) revealed 17 spots which were differentially expressed in pre-eclamptic and normal placentas. MALDI TOF/TOF mass analysis verified 11 of these 17 spots. Among them, four proteins (chloride intracellular channel 3, apolipoprotein A-I, transthyretin (TTR) and protein disulphide isomerase) were increased, while seven (e.g. peroxiredoxin 2 and 3, Hsc 70) showed decreased expression in pre-eclamptic versus control placentas. The down-regulated proteins with anti-oxidant activity (peroxiredoxins) and altered expression of stress-response proteins (Hsc, protein disulphide isomerase) could play an important role in the pathogenesis of pre-eclampsia (Gharesi-Fard et al. 2010).

The syncytiotrophoblast layer is the site of many important placental functions necessary for fetal growth and development, including nutrient and gas exchange, and synthesis of hormones (Eaton et al. 1993). Many studies have shown that hypoxia inhibits cytotrophoblast differentiation and cell fusion. Hypoxia-induced responses of syncytialization were examined in a study by Hu et al. (Hu et al. 2007). They showed that 20 proteins were significantly differentially expressed in hypoxia compared with normoxic cell lines. In response to hypoxia, three antioxidants (peroxiredoxin 1 and 2 as well as 1-Cys peroxiredoxin) were down-regulated and two proteins involved in the glycolysis pathway were up-regulated. Furthermore, the expression levels of annexin A2 and annexin A5 were increased. They also found that the expression of proteins that regulate cellular oxidative stress, signal transduction, protein folding and degradation, cell mobility and cytoskeletal structure formation were altered.

Systematic characterization of the early pregnancy maternal serum proteome profile of asymptomatic women who subsequently developed pre-eclampsia demonstrated a distinct proteome profile compared with that in women with clinical disease. Differentially expressed proteins included placental, vascular,
transport, matrix and acute-phase proteins. Expression of angiogenic and anti-
angiogenic proteins was not significantly different between the study groups. The 
results indicate that matrix and structural proteins may play a more important role 
in the early placentation process than angiogenic proteins (Rasanen et al. 2010).

2.6 Doppler ultrasonography in assessment of uterine and 
placental haemodynamics

2.6.1 Methodological aspects

Doppler ultrasonography is a widely used non-invasive method to assess maternal 
and fetal haemodynamics. The vessel of interest is identified by colour-Doppler 
ultrasonography, and then pulsed wave Doppler ultrasonography (single transducer, 
which transmits and receives the ultrasound waves in pulses) is used to obtain blood 
velocity waveforms for analysis. Blood flow in the uterine, umbilical and fetal 
arteries is most often semiquantitatively described by calculating the pulsatility 
index (PI = (peak systolic velocity minus end diastolic velocity)/time-averaged 
maximum velocity over the cardiac cycle) or resistance index (RI = (peak systolic 
velocity minus end diastolic velocity)/peak systolic velocity) values (Nelson & 
Pretorius 1988, Burns 1993). These angle-independent indices reflect downstream 
vascular impedance.

Safety aspects

Ultrasound is a mechanical energy in which a pressure wave travels through 
tissue. The physical effects of ultrasound are generally categorized as: thermal 
(heating of tissue as ultrasound is absorbed) and non-thermal effects (cavitation, 
i.e. the formation of gas bubbles at a high negative pressure and radiation pressure 
streaming). Hyperthermia has been shown to have teratogenic effects in animal 
However, many animal studies have shown no harmful pregnancy or post-natal 
effects in exposed animals (Tarantal & Hendrickx 1989a, Tarantal & Hendrickx 
1989b). In humans, no significant deleterious consequences of multiple sonographic 
studies performed during the second and third trimesters were noted after follow-
up of 8 years (Newnham et al. 2004). It is impossible, however, to measure the 
actual in situ exposure in human fetuses and therefore a measure of thermal output 
was developed. The thermal index (TI) serves as an approximation of the thermal
risk being produced during an ultrasonographic examination and it is calculated automatically by the equipment in real-time. A TI of less than one is generally accepted as safe. The World Federation for Ultrasound in Medicine and Biology has concluded that exposure producing a maximum temperature elevation of no more than 1.5 °C above normal physiological levels is acceptable (Barnett et al. 2000). In a recent study by Sheiner et al. (Sheiner et al. 2007) it was reported that first trimester routine sonographic examination is associated with a negligible rise in TI. Non-thermal, or mechanical, effects have also been recognized as a potential mechanism for harm during ultrasonographic examinations and they refer to a relationship between interfaces, such as ultrasound waves and gas bubbles, which reside within tissue (Stratmeyer et al. 2008). A mechanical index (MI) is used to approximate mechanical strain. Analogous to the TI, a value of less than one is considered as safe.

2.6.2 Haemodynamic and endometrial predictors of uterine receptivity

In IVF and ICSI, the great majority of good-quality embryos fail to implant. Although it is well known that there are multiple factors important for successful implantation, the success rate has been predicted by evaluating uterine haemodynamics and the endometrium.

Uterine circulation

The two main uterine arteries (UtAs) originate from the internal iliac arteries and enter the uterus above the cervix at the level of the isthmus uteri. In the myometrium, the uterine arteries form a network of arcuate arteries, which spread into the outer third of the myometrium and give off the radial arteries. The radial arteries are called spiral arteries when they reach the uterine endometrium.

It was first reported in 1988 that impaired perfusion of the UtAs may be a cause of infertility and may be related to unsuccessful IVF (Goswamy et al. 1988). Several studies have shown that an increase in uterine vascular resistance with a concomitant decrease in uterine blood flow significantly reduce the likelihood of pregnancy during IVF treatment (Coulam et al. 1994, Serafini et al. 1994, Cacciatore et al. 1996, Zaidi et al. 1996, Singh et al. 2011). Although mean PI and RI values in the UtAs are lower during successful than in failed IVF cycles, the overlap of values is considerable. Puerto et al. (Puerto et al. 2003) reported that women pregnant after IVF had endometrial thicknesses and UtA PI values
similar to those in women with similar embryo scoring who did not conceive. The significant differences between these two groups were that in women who conceived the endometrium was homogeneous and hyperechogenic, and the early diastolic notch in the UtA was absent. In addition, Hoozemans et al. (Hoozemans et al. 2008) found no statistically significant differences in UtA PI values during successful versus unsuccessful IVF cycles.

Many studies have been focused on the main external UtAs, but some investigators have studied blood velocity waveforms of the small endometrial vessels to assess endometrial perfusion. During hormone treatment for oocyte donation, Achiron et al. (Achiron et al. 1995) demonstrated a significant increase in endometrial blood flow and a decrease in PI correlated with the hormonal profile. An increase in endometrial diastolic blood flow during the proliferative phase was noted. Absent subendometrial and intraendometrial blood flow on the day of hCG administration or embryo transfer has been associated with decreased implantation and pregnancy rates in IVF (Zaidi et al. 1995, Chien et al. 2002, Singh et al. 2011). However, in a study by Schild et al. (Schild et al. 2001), absent spiral artery flow was not linked to implantation rate. In addition, Isaksson et al. (Isaksson et al. 2003) reported similar spiral artery PI and peak systolic velocity values during successful versus unsuccessful IVF cycles among women with unexplained or tubal infertility. Altogether, the results pertaining to uterine haemodynamics in the assessment of the success of IVF and ICSI treatment are conflicting.

**Endometrial properties**

Endometrial thickness is defined as the distance between the echogenic interfaces of the myometrium in the central sagittal axis of the uterine body. It reflects endometrial growth during the menstrual cycle. While some ultrasonographic studies have demonstrated that a substantial proportion of women with successful implantation have had a thicker endometrium (Noyes et al. 1995, Singh et al. 2011) with more organized morphology (Serafini et al. 1994) than in women without conception, many studies have failed to confirm these results (Dickey et al. 1992, Khalifa et al. 1992, Oliveira et al. 1993). Reported ranges of mean endometrial thicknesses in conception and non-conception cycles are virtually the same, being 8.6–11.8 mm and 8.6–11.9 mm (Friedler et al. 1996). At present, insufficient data exist to show a linear correlation between endometrial thickness and the likelihood of conception. A minimum endometrial thickness of 6 mm has been stated to be required for implantation in cases of insemination (Gonen et al. 1991) and in IVF.
(Singh et al. 2011). In recent studies, endometrial thicknesses have varied between 5 and 8 mm in cases of successful implantation, when measured during the late proliferative to early luteal phase (Singh et al. 2011).

Welker et al. (Welker et al. 1989) suggested that the endometrial pattern (texture, reflectivity), as assessed by ultrasonography, may be a predictor of successful implantation following IVF-ET. Endometrial pattern is defined in terms of relative echogenicity of the endometrium when compared with adjacent myometrium. A triple-line, multilayered pattern is more frequently, but not exclusively, associated with conception cycles and thus could reflect endometrial receptivity (Welker et al. 1989, Dickey et al. 1992, Serafini et al. 1994). However, a non-multilayered endometrial pattern does not exclude the possibility of pregnancy (Dickey et al. 1992, Serafini et al. 1994). Moreover, al-Shawaf et al. (al-Shawaf et al. 1993) showed that classification of endometrial pattern did not significantly improve the ability to predict conception following assisted reproduction treatment. The limited value of endometrial pattern in the evaluation of endometrial receptivity may be due to the fact that sonographic assessment of endometrial morphology is subjective, depending on the observer and the ultrasound equipment.

2.6.3 Ultrasonographic assessment of placentation

Normal pregnancy

Low end-diastolic blood flow velocity and an early diastolic notch in UtA blood flow velocity waveforms are characteristic in the non-pregnant state and during the first trimester. During normal pregnancy there is an increase in diastolic blood flow, reflected in decreased blood flow velocity ratios and gradual disappearance of the diastolic notch in the UtAs (Schulman et al. 1987, Harrington & Campbell 1992). These alterations, reflecting low-resistance uteroplacental circulation, occur in normal pregnancies generally by 18–20 weeks of gestation, and at the latest by 24–26 weeks (Bower et al. 1993). Jurkovic et al. (Jurkovic et al. 1991) and Jauniaux et al. (Jauniaux et al. 1992) showed that UtA PI decreased from a mean value of 2.0 to 1.3 between 8 and 18 weeks of gestation. The increase in blood supply to the intervillous space is mainly a result of successful trophoblastic invasion, and remodelling of spiral arteries. Impedance to blood flow in the uterine and spiral arteries decreases with advancing pregnancy, while in pregnancies complicated by pre-eclampsia or IUGR vascular impedance is increased (Trudinger et al. 1985, van Zalen-Sprock et al. 1994).

There is some evidence that ovulation induction can affect UtA blood flow
in early pregnancy (Dickey & Hower 1995). Between 4–9 weeks of pregnancy, UtA volume blood flow was significantly higher in patients after controlled ovarian hyperstimulation compared with those who conceived spontaneously. Until 9 weeks of pregnancy, blood flow velocities were significantly increased and RI values significantly decreased in patients treated with clomiphene citrate plus gonadotrophins compared with those who conceived spontaneously or used clomiphene citrate alone. After 10 weeks of pregnancy, these differences were no longer present (Dickey & Hower 1995).

Pathological pregnancies

The preliminary observations of increased UtA vascular impedance in pregnancies complicated by pre-eclampsia and IUGR led to a number of studies in which the possibility to predict those pregnancies destined to develop pre-eclampsia or IUGR was assessed. These studies showed that women with increased vascular impedance in UtA blood flow have an increased risk of developing pre-eclampsia (Harrington et al. 1995, Papageorghiou et al. 2004, Yu et al. 2005, Spencer et al. 2006) and, in particular, severe pre-eclampsia with IUGR (Papageorghiou et al. 2001). The prediction rate as regards pre-eclampsia, using Doppler ultrasonography during the second trimester of pregnancy, is about 40% and for severe pre-eclampsia requiring delivery at <34 gestational weeks about 80%, with a 5% false screen-positive rate, and detection can be further increased by using Doppler ultrasonography in combination with maternal medical history (Papageorghiou et al. 2005, Yu et al. 2005, Plasencia et al. 2007). Women with normal impedance to flow in the UtAs have only a low risk of developing obstetric complications related to uteroplacental insufficiency (Papageorghiou et al. 2002).

Over the last decade, interest in UtA Doppler ultrasonographic assessment in predicting pre-eclampsia has shifted from the second to the first trimester. Many studies have shown that UtA impedance is already increased at 11–14 weeks of gestation in women who subsequently develop pre-eclampsia (Papageorghiou et al. 2005, Plasencia et al. 2007). Sensitivity as regards predicting severe or early-onset disease is much higher (82%) than is that for mild or late-onset disease (30%) and combined screening using maternal variables and UtA Doppler ultrasonography resulted in improved detection of late-onset disease from 30 to 50% (Papageorghiou et al. 2005, Yu et al. 2005, Plasencia et al. 2007).
2.7 Low-dose acetylsalicylic acid

Acetylsalicylic acid (aspirin) is the first-discovered member of the class of drugs known as non-steroidal anti-inflammatory drugs (NSAIDs), which have analgesic, anti-inflammatory and antipyretic properties. The mechanism of action of aspirin is dose-dependent. High doses (>325 mg/day) mainly relieve pain and inflammatory reactions; lower doses prevent platelet aggregation and vasoconstriction.

2.7.1 Mechanism of action

Aspirin suppresses the production of prostaglandins and thromboxane by irreversibly inhibiting the enzyme COX. It acts as an acetylating agent where an acetyl group is covalently attached to a serine residue (Ser-529) in the active site of COX enzymes (Figure 3). This makes aspirin different from other NSAIDs, which are reversible inhibitors of COX enzymes (Vane 1971). Low-dose aspirin irreversibly blocks thromboxane A\(_2\) synthesis in the platelets, which lack the ability to synthesize COX. The block induced by aspirin cannot be repaired during their life-span (approximately 8–10 days) (Patrono 1994). In contrast, endothelial cells are able to re-synthesize COX after being exposed to aspirin and prostacyclin synthesis is thus re-established in vessel endothelium (Willis 1974).
Fig. 3. Biosynthesis of prostanoids and the action site of aspirin.

2.7.2 Aspirin in IVF and ICSI treatment

Aspirin, in theory, could enhance blood flow and thus improve the success rate in IVF treatment. Wada et al. (Wada et al. 1994) first found that low-dose aspirin therapy (150–300 mg/day) improves uterine blood flow and pregnancy rates in women with impaired uterine perfusion during assisted conception. Since then the effects of low-dose aspirin therapy during IVF and ICSI have been investigated in numerous studies (Hasegawa et al. 1998, Rubinstein et al. 1999, Urman et al. 2000, Lok et
al. 2004, Waldenstrom et al. 2004, Moini et al. 2007, Frattarelli et al. 2008, Dirckx et al. 2009, Lambers et al. 2009a). The first randomized study, by Rubinstein et al. (Rubinstein et al. 1999), revealed significantly higher implantation and clinical pregnancy rates, as well as significantly lower uterine artery PI values on the day of hCG administration in aspirin-treated (100 mg/day) women with tubal infertility compared with the placebo group. Moreover, the largest study (open and quasi-randomized), by Waldenstrom et al. (Waldenstrom et al. 2004), (Table 2), which included 1022 patients and a total of 1380 consecutive cycles with a short regimen protocol showed a slightly higher birth rate in aspirin-treated (75 mg/day) patients compared with the no-treatment group. In contrast, all the other studies (Urman et al. 2000, Moini et al. 2007, Dirckx et al. 2009, Lambers et al. 2009a) did not show differences in implantation or pregnancy rates between aspirin- and placebo-treated women, not even among poor responders (Lok et al. 2004, Frattarelli et al. 2008).

A list of randomized studies in which the effect of low-dose aspirin on clinical pregnancy rate has been evaluated is presented in Table 2. The results concerning aspirin treatment in frozen-thawed embryo transfers and oocyte donation patients are contradictory (Wada et al. 1994, Weckstein et al. 1997, Check et al. 1998a).

Five systematic reviews or meta-analyses have been performed to evaluate the effect of low-dose aspirin on the outcome parameters in IVF and ICSI (Gelbaya et al. 2007, Khairy et al. 2007, Poustie et al. 2007, Ruopp et al. 2008, Groeneveld et al. 2011). Of these, only the study by Ruopp et al. (Ruopp et al. 2008) showed an increase in clinical pregnancy rate in the aspirin group compared with the placebo group. These reviews/meta-analyses and their main outcome measures are summarized in Table 3.

Rubenstein et al. (Rubenstein et al. 1999) found that low-dose aspirin improved blood flow in the uterine and ovarian arteries during IVF cycles in women with tubal infertility. However, recent studies by Lok et al. (Lok et al. 2004) and Lambers et al. (Lambers et al. 2009a) could not confirm these findings, even in women with poor prognosis.
Table 2. Randomized prospective studies in which the effect of low-dose aspirin therapy on clinical pregnancy rate in fresh IVF and ICSI treatments has been evaluated.

<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Participants</th>
<th>Period of treatment</th>
<th>Clinical pregnancy rate/cycles initiated</th>
<th>Treatment group n (%)</th>
<th>Control group n (%)</th>
<th>p-value or OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubinstein et al. 1999</td>
<td>298</td>
<td>Tubal factor</td>
<td>Preceding cycle until 12 weeks’ gestation</td>
<td>67/149 (45)</td>
<td>42/149 (28)</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Urman et al. 2000</td>
<td>279</td>
<td>Male factor</td>
<td>First FSH day until 6 weeks’ gestation</td>
<td>55/139 (39)</td>
<td>59/136 (43)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Bordes et al. 2003*</td>
<td>138</td>
<td>Unselected</td>
<td>Preceding cycle until FHB seen in US</td>
<td>27/69 (39)</td>
<td>15/69 (22)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Lentini et al. 2003*</td>
<td>84</td>
<td>Unselected</td>
<td>One month before GnRH until pregnancy test</td>
<td>13/42 (31)</td>
<td>10/42 (24)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Lok et al. 2004</td>
<td>60</td>
<td>Poor responders</td>
<td>Preceding cycle until hCG administration</td>
<td>1/30 (3.3)</td>
<td>2/30 (6.6)</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>Waldenström et al. 2004*</td>
<td>1380</td>
<td>Unselected</td>
<td>ET day until pregnancy test</td>
<td>249/703 (35)</td>
<td>203/677 (30)</td>
<td>1.3 (1.0-1.6)</td>
<td></td>
</tr>
<tr>
<td>Van Dooren et al. 2004*</td>
<td>81</td>
<td>First IVF cycle</td>
<td>Preceding cycle until 10 weeks’ gestation</td>
<td>31/85 (36)</td>
<td>29/85 (34)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Duvan et al. 2006</td>
<td>81</td>
<td>Unselected</td>
<td>ET day until pregnancy test</td>
<td>11/41 (27)</td>
<td>14/40 (35)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Moini et al. 2007</td>
<td>145</td>
<td>Unselected</td>
<td>Preceding cycle until 12 weeks’ gestation</td>
<td>33/72 (45)</td>
<td>24/73 (32)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Dirckx et al. 2009</td>
<td>201</td>
<td>Unselected</td>
<td>Preceding cycle until FHB seen in US</td>
<td>31/97 (32)</td>
<td>30/96 (31)</td>
<td>1.03 (0.57-1.89)</td>
<td></td>
</tr>
<tr>
<td>Lambers et al. 2009*</td>
<td>169</td>
<td>Non-tubal, previous failed conception</td>
<td>Preceding cycle until 12 weeks’ gestation</td>
<td>26/85 (31)</td>
<td>28/84 (35)</td>
<td>0.677</td>
<td></td>
</tr>
</tbody>
</table>

*Abstract, *Short regimen and open study, FHB=fetal heart beat, US=ultrasonography, ET=embryo transfer, ns=not significant (exact level of significance not given in the original publication)
Table 3. Meta-analyses and systematic reviews in which the effect of low-dose aspirin therapy on outcome parameters in IVF and ICSI patients have been evaluated.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of patients/trials</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelbaya et al. 2007</td>
<td>2151/6</td>
<td></td>
</tr>
<tr>
<td>PR/ET</td>
<td>1.09 (0.92-1.29)</td>
<td></td>
</tr>
<tr>
<td>LB/ET</td>
<td>1.08 (0.83-1.40)</td>
<td></td>
</tr>
<tr>
<td>Miscarriage/PR</td>
<td>1.17 (0.84-1.63)</td>
<td></td>
</tr>
<tr>
<td>Ectopic pregnancy/PR</td>
<td>1.22 (0.34-4.38)</td>
<td></td>
</tr>
<tr>
<td>Khairy et al. 2007</td>
<td>1241/7</td>
<td></td>
</tr>
<tr>
<td>PR/cycles initiated</td>
<td>1.11 (0.95-1.31)</td>
<td></td>
</tr>
<tr>
<td>LB/cycles initiated</td>
<td>0.94 (0.64-1.39)</td>
<td></td>
</tr>
<tr>
<td>Miscarriage/cycles initiated</td>
<td>1.06 (0.53-2.11)</td>
<td></td>
</tr>
<tr>
<td>Ectopic pregnancy/cycles initiated</td>
<td>2.24 (0.70-7.24)</td>
<td></td>
</tr>
<tr>
<td>Poustie et al. 2007</td>
<td>1449/10</td>
<td></td>
</tr>
<tr>
<td>PR/cycles initiated</td>
<td>1.09 (0.83-1.43)</td>
<td></td>
</tr>
<tr>
<td>LB/cycles initiated</td>
<td>0.94 (0.63-1.39)</td>
<td></td>
</tr>
<tr>
<td>Miscarriage/cycles initiated</td>
<td>1.17 (0.61-2.27)</td>
<td></td>
</tr>
<tr>
<td>Ectopic pregnancy/cycles initiated</td>
<td>2.24 (0.69-7.22)</td>
<td></td>
</tr>
<tr>
<td>Ruopp et al. 2008</td>
<td>2801/9</td>
<td></td>
</tr>
<tr>
<td>PR/ET</td>
<td>1.15 (1.03-1.29)</td>
<td></td>
</tr>
<tr>
<td>Miscarriage/ET</td>
<td>1.17 (0.84-1.63)</td>
<td></td>
</tr>
<tr>
<td>Groeneveld et al. 2010*</td>
<td>1119/6</td>
<td></td>
</tr>
<tr>
<td>PR/cycles initiated</td>
<td>0.86 (0.67-1.10)</td>
<td></td>
</tr>
<tr>
<td>LB/cycles initiated</td>
<td>0.85 (0.64-1.10)</td>
<td></td>
</tr>
<tr>
<td>Miscarriage/cycles initiated</td>
<td>1.20 (0.61-2.30)</td>
<td></td>
</tr>
</tbody>
</table>

PR=clinical pregnancy rate, ET=embryo transfer, LB=live birth rate, *individual patient data

2.7.3 Aspirin in prevention of hypertensive pregnancy complications

In pre-eclampsia, inadequate placental perfusion and ischaemia lead to endothelial dysfunction (Redman et al. 1978). At three months of pregnancy Beaufils et al. (Beaufils et al. 1985) randomized 102 women with an increased risk of pre-eclampsia and/or IUGR on the basis of their past obstetric history to receive dipyridamole (300 mg/day) and low-dose aspirin (150 mg/day) or no treatment until delivery. The incidences of pre-eclampsia and fetal loss were significantly lower, and fetal as well as placental weights significantly higher in the treatment group than in the controls. They concluded that inhibition of platelet aggregation,
when used early in pregnancy, may have a significant protective effect against pre-eclampsia and IUGR in a high risk population (Beaufils et al. 1985). Since then, numerous trials have been carried out to investigate the usefulness of low-dose aspirin alone or combined with other antiplatelet agents in prevention of pre-eclampsia and IUGR. However, the results of randomized trials are contradictory. While many early small studies showed that aspirin significantly lowered the incidence of pre-eclampsia (Beaufils et al. 1985, Wallenburg et al. 1986, Schiff et al. 1989), the results of subsequent large randomized trials did not confirm this (Sibai et al. 1993, CLASP 1994, ECPPA 1996, Caritis et al. 1998, Golding 1998, Rotchell et al. 1998, Subtil et al. 2003). The largest randomized study (CLASP 1994), with 9364 women, demonstrated 12% reduction (statistically not significant) in the incidence of pre-eclampsia in aspirin-treated (60 mg/day) compared with placebo-treated patients. There were no significant differences in the incidence of IUGR, stillbirths or neonatal death. Aspirin did, however, reduce the likelihood of preterm delivery (CLASP 1994). In these large randomized studies, low-dose aspirin therapy was started mainly in the late second trimester or during the third trimester. Some studies have shown that low-dose aspirin therapy may be beneficial when started earlier, during the late first trimester (Vainio et al. 2002, Ebrashy et al. 2005). These results are in agreement with those in a recent meta-analysis by Bujold et al. (Bujold et al. 2010), who found that low-dose aspirin, when started at 16 weeks of pregnancy or earlier, was associated with a significant reduction of pre-eclampsia and IUGR. In addition, a recent study by Lambers et al. (Lambers et al. 2009b) demonstrated that the incidence of hypertensive pregnancy complications was significantly lower among aspirin-treated (100 mg/day) IVF and ICSI patients with non-tubal infertility and previous implantation failure compared with placebo-treated women, when medication was started in the previous cycle. In an individual patient data meta-analysis carried out by Askie et al. (Askie et al. 2007), no statistically significant differences were found between aspirin-treated and control subjects when medication was started before 20 gestational weeks or beyond. On the other hand, they reported a 10% overall decrease in the risk of pre-eclampsia in the aspirin group compared with the controls. A very recent meta-analysis carried out by Trivedi (Trivedi 2011) showed a 21% reduction in the incidence of pre-eclampsia brought about by low-dose aspirin in a high-risk group, but the overall incidence of pre-eclampsia and the incidence in a low-risk group did not differ between aspirin- and placebo-treated women. The meta-analyses and systematic reviews in which the effects of low-dose aspirin on placenta-related pregnancy complications have been evaluated are listed in Table 4.
Table 4. Meta-analyses and systematic reviews in which the effectiveness of antiplatelet therapy, mostly low-dose aspirin (50–150 mg/day), on prevention of pre-eclampsia, IUGR, prematurity and perinatal mortality has been evaluated.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of patients/tabs</th>
<th>Pre-eclampsia</th>
<th>IUGR</th>
<th>Prematurity &lt;34 weeks</th>
<th>PNM</th>
<th>Treatment initiation (gestational weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leitich <em>et al.</em> 1997&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13234/13</td>
<td>–</td>
<td>0.82 (0.72-0.93)</td>
<td>–</td>
<td>ns</td>
<td>12-32</td>
</tr>
<tr>
<td>ASA 50-80 mg/day</td>
<td>–</td>
<td>0.87 (0.76-0.99)</td>
<td>–</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA 100-150 mg/day</td>
<td>–</td>
<td>0.36 (0.22-0.59)</td>
<td>–</td>
<td>0.40 (0.16-0.97)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA initiated &lt; 17 weeks</td>
<td>–</td>
<td>0.35 (0.21-0.58)</td>
<td>–</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coomarasamy <em>et al.</em> 2001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>498/5</td>
<td>0.55 (0.32-0.95)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>17-24</td>
</tr>
<tr>
<td>Coomarasamy <em>et al.</em> 2003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12416/14</td>
<td>0.86 (0.76-0.96)</td>
<td>ns</td>
<td>0.86 (0.79-0.94)</td>
<td>0.79 (0.64-0.96)</td>
<td>12-32</td>
</tr>
<tr>
<td>Ruano <em>et al.</em> 2005</td>
<td>3359/822</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-risk group</td>
<td>16700/5</td>
<td>ns</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>High-risk group&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16898/17</td>
<td>0.87 (0.79-0.96)</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duley <em>et al.</em> 2007</td>
<td>3756/59</td>
<td>0.83 (0.77-0.89)</td>
<td>0.90 (0.83-0.98)</td>
<td>0.92 (0.89-0.97)</td>
<td>0.86 (0.76-0.98)</td>
<td>12-36</td>
</tr>
<tr>
<td>Askie <em>et al.</em> 2007</td>
<td>32217/31</td>
<td>0.90 (0.84-0.97)</td>
<td>ns</td>
<td>0.90 (0.83-0.98)</td>
<td>ns</td>
<td>12→</td>
</tr>
<tr>
<td>Bujold <em>et al.</em> 2009&lt;sup&gt;e&lt;/sup&gt;</td>
<td>13179</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA initiated ≤ 16 weeks</td>
<td>–</td>
<td>0.48 (0.33-0.68)</td>
<td>0.51 (0.28-0.92)</td>
<td>–</td>
<td>–</td>
<td>12-16</td>
</tr>
<tr>
<td>ASA initiated 17-19 weeks</td>
<td>ns</td>
<td>ns</td>
<td>–</td>
<td>–</td>
<td></td>
<td>17-19</td>
</tr>
<tr>
<td>ASA initiated ≥ 20 weeks</td>
<td>ns</td>
<td>ns</td>
<td>–</td>
<td>–</td>
<td></td>
<td>20→</td>
</tr>
<tr>
<td>Bujold <em>et al.</em> 2010</td>
<td>11348/27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA initiated ≤ 16 weeks</td>
<td>764</td>
<td>0.47 (0.34-0.65)</td>
<td>0.44 (0.30-0.65)</td>
<td>0.22 (0.10-0.49)</td>
<td>–</td>
<td>12-16</td>
</tr>
<tr>
<td>ASA initiated &gt; 16 weeks</td>
<td>10584</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td>18-36</td>
</tr>
<tr>
<td>Trivedi 2011</td>
<td>28237/19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-risk group</td>
<td>16550</td>
<td>ns</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>12-32</td>
</tr>
<tr>
<td>High-risk group&lt;sup&gt;f&lt;/sup&gt;</td>
<td>11687</td>
<td>0.79 (0.65-0.97)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>12-32</td>
</tr>
</tbody>
</table>

IUGR=intrauterine growth restriction, PNM=perinatal mortality, *in 7 studies out of 11 all the participants started medication at ≤ 24 weeks of pregnancy, *study included patients at high risk of pre-eclampsia due to abnormal uterine artery Doppler ultrasonographic results in the second trimester, *study included patients with historical risk factors of pre-eclampsia, *study included patients with previous pre-eclampsia, diabetes mellitus, renal disease or essential hypertension, *study included patients with abnormal uterine artery Doppler ultrasonographic results in the first or second trimester, high-risk conditions (essential hypertension and diabetes mellitus), positive angiotensine sensitivity, positive rollover test or repeated spontaneous abortions, ns = not significant, – = not evaluated
2.7.4 Adverse effects

Prenatal use of NSAIDs/aspirin has been reported to increase the risk of miscarriage. An interview-based cohort study by Li et al. (Li et al. 2003) showed that the incidence of miscarriage was 1.6- and 1.8-fold higher in aspirin and NSAIDs users, respectively, compared with non-users. In particular, the use of medication at the time of conception or for longer than a week was associated with an elevated miscarriage risk. Nielsen et al. (Nielsen et al. 2001) observed in their register study that NSAIDs can increase the risk of miscarriage up to almost 7-fold.

Aspirin crosses the placenta and it has been associated with an increased risk of gastroschisis (Werler et al. 1992, Kozer et al. 2002). Aspirin use has not been linked to any other congenital malformations (Czeizel et al. 2000, Nielsen et al. 2001, Kozer et al. 2002, Norgard et al. 2005). In meta-analyses by Werler et al. (Werler et al. 1992) and Kozer et al. (Kozer et al. 2002), the incidence of gastroschisis has been shown to be 2.4- to 2.7-fold greater in mothers with aspirin exposure compared with controls. The authors found no association between several other over-the-counter NSAIDs, including ibuprofen, and gastroschisis. There is evidence, however, that the use of pseudoephedrine or acetaminophen during organogenesis is also associated with gastroschisis, suggesting that maternal disease could contribute to the observed association (Werler et al. 1992).

Patency of the ductus arteriosus (DA) is prostaglandin-dependent. Exposure to 100 mg of aspirin daily from 18 gestational weeks onwards until delivery did not cause premature closure of the DA (Grab et al. 2000). The risk of DA constriction due to use of low-dose aspirin is probably small or nonexistent.

Sibai et al. (Sibai et al. 1993) reported an increased risk of placental abruption in women who used low-dose aspirin compared with women using placebo. In contrast, a meta-analysis of 11 studies did not reveal a statistically significant difference in placental abruption rate between exposed and non-exposed women (Hauth et al. 1995a).

Large meta-analyses have shown that daily use of low-dose aspirin from the second trimester until delivery does not significantly increase bleeding complications during pregnancy or postpartum (Leitich et al. 1997, Duley et al. 2001, Askie et al. 2007).
3 Aims

By decreasing platelet aggregation and inhibiting vasoconstriction, low-dose aspirin may enhance uterine and ovarian blood flow and tissue perfusion and thus improve the results of IVF and ICSI treatments. In this randomized, placebo-controlled and double-blind study, the first hypothesis was that low-dose aspirin therapy (100 mg daily) would improve the outcomes of IVF- and ICSI-treated patients and enhance uterine receptivity when medication is started concomitantly with controlled ovarian hyperstimulation. The specific aims of the study were:

1. To investigate whether low-dose aspirin improves ovarian responsiveness, clinical pregnancy rate and live-birth rate in unselected women undergoing fresh IVF or ICSI embryo transfer (I, V).
2. To determine whether uterine circulation and endometrial thickness are improved at the time of embryo transfer by low-dose aspirin therapy (II).

In IVF and ICSI pregnancies the placental protein pattern is altered in the first and second trimesters. Similar alterations are seen in pre-eclampsia and in fetal growth restriction, which are characterized by abnormal trophoblast invasion into maternal spiral arteries and high uterine artery vascular impedance detected by Doppler ultrasonography. The second hypothesis was that in infertile patients, IVF and ICSI treatment modifies the placentation process as demonstrated by altered maternal serum placental protein levels, and low-dose aspirin therapy, initiated before implantation, affects placentation, resulting in a normalized maternal serum placental protein pattern. More specific aims were:

1. To determine whether the maternal serum placental proteome profile is different in IVF and ICSI pregnancies compared with spontaneous pregnancies and whether it is modified by low-dose aspirin (III).
2. To investigate whether low-dose aspirin therapy modifies the uteroplacental circulation and reduces pregnancy-induced placental complications (IV, V).
4 Material and methods

4.1 Study subjects

The patients were recruited from Departments of Obstetrics and Gynaecology at the Universities of Oulu, Kuopio and Tampere, and from The Family Federation Infertility Clinic in Oulu during the years 2001–2007. After patient recruitment for (multi-centre) Study I was completed (adequate sample size based on power calculation), we continued recruiting patients at Oulu University Hospital for Studies II and IV in order to collect the required amount of patients based on sample size calculations. The reason that patient collection for Studies II and IV was continued only at Oulu University Hospital and The Family Federation Infertility Clinic, Oulu was that these two studies required ultrasonographic examinations and in order to minimize methodological errors (inter-observer error), the ultrasonographic examinations were performed by a single investigator. In Study II there were 91 patients who were included in Study I and among whom ultrasonographic examination on the day of ET was performed. Demographic characteristics of the patients included and not included in Study II, from the patient cohort of Study I, are presented in Table 5. An additional 31 patients were recruited to Study II and randomized following the original randomization protocol to either the low-dose aspirin or the placebo group. The person performing ultrasonography was blind to the treatment arm.

In Study IV, there were 146 patients who were also included in Study I. Twenty-eight of these women who became pregnant underwent ultrasonographic examinations four times during the first half of pregnancy. Demographic characteristics of the patients included and not included in Study IV, from the patient cohort of Study I, are presented in Table 6. An additional 9 pregnant patients for Study IV were collected from the study cohort that was recruited and randomized after Study I and their treatment codes were deciphered after their deliveries. All the data presented in Studies I, II and IV were collected and the ultrasonographic parameters calculated prior to opening of the randomization code. After six and a half years of recruitment a total of 487 patients were collected and included in analysis of the follow-up study (Study V).

Inclusion criteria were: I) age <40 years, II) <4 previous instances of ovarian stimulation, and III) no contraindications for aspirin. The study subjects were from all aetiological categories of infertility and all of them underwent IVF or ICSI treatment. The study protocols were randomized, placebo-controlled and double-blind and are shown in Table 7.
The Ethics Committees in each institution approved the study protocols for Studies I and V and the local Ethics Committee of Oulu University approved all the study protocols (I–V).

Table 5. Demographic characteristics of the patients included and not included in Study II from the patient cohort of Study I.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients included in Study II from Study I (n=91/184)</th>
<th>Patients not included in Study II from Study I (n=93/184)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31.3±4.1</td>
<td>31.9±4.2</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.1±4.3</td>
<td>24.8±4.1</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>4.3±2.7</td>
<td>3.9±2.5</td>
</tr>
<tr>
<td>Aetiology of infertility % (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male factor</td>
<td>26 (23)</td>
<td>30 (28)</td>
</tr>
<tr>
<td>Tubal</td>
<td>14 (13)</td>
<td>20 (18)</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>22 (20)</td>
<td>22 (20)</td>
</tr>
<tr>
<td>Other female</td>
<td>9 (8)</td>
<td>8 (7)</td>
</tr>
<tr>
<td>Unexplained</td>
<td>26 (24)</td>
<td>20 (18)</td>
</tr>
<tr>
<td>Mixed</td>
<td>3 (3)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Pregnancy history % (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients with previous pregnancy</td>
<td>39 (35)</td>
<td>42 (39)</td>
</tr>
<tr>
<td>Total no. of pregnancies</td>
<td>32</td>
<td>37</td>
</tr>
<tr>
<td>Live births</td>
<td>66 (21)</td>
<td>62 (23)</td>
</tr>
<tr>
<td>Spontaneous abortions</td>
<td>34 (11)</td>
<td>38 (14)</td>
</tr>
</tbody>
</table>

Data are given as mean±SD.
Table 6. Demographic characteristics of the patients included and not included in Study IV from the patient cohort of Study I.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients included in Study IV from Study I (n=146/184)</th>
<th>Patients not included in Study IV from Study I (n=38/184)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>33.0±4.1</td>
<td>31.7±4.0</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.6±4.3</td>
<td>23.8±4.5</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>4.2±2.6</td>
<td>4.8±2.9</td>
</tr>
<tr>
<td>Aetiology of infertility % (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male factor</td>
<td>20 (29)</td>
<td>26 (10)</td>
</tr>
<tr>
<td>Tubal</td>
<td>14 (21)</td>
<td>18 (7)</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>21 (30)</td>
<td>24 (9)</td>
</tr>
<tr>
<td>Other female</td>
<td>10 (14)</td>
<td>8 (3)</td>
</tr>
<tr>
<td>Unexplained</td>
<td>29 (43)</td>
<td>21 (8)</td>
</tr>
<tr>
<td>Mixed</td>
<td>6 (9)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>Pregnancy history % (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients with previous pregnancy</td>
<td>33 (49)</td>
<td>29 (11)</td>
</tr>
<tr>
<td>Total no. of pregnancies</td>
<td>86</td>
<td>13</td>
</tr>
<tr>
<td>Live births</td>
<td>34 (30)</td>
<td>38 (5)</td>
</tr>
<tr>
<td>Spontaneous abortions</td>
<td>66 (56)</td>
<td>62 (8)</td>
</tr>
</tbody>
</table>

Data are given as mean±SD.

4.2 Randomization

Eligible patients who signed a written informed consent document were randomly allocated on the first day of gonadotrophin stimulation to receive 100 mg oral aspirin or placebo daily in one dose until menstruation or a negative pregnancy test result. Women who became pregnant continued the medication until delivery. Randomization was carried out by means of computer-generated random numbers in blocks of four by the pharmacist (third-party administrator) at Oulu University Hospital. Concealment of allocation was achieved by using opaque sealed envelopes. Blinding of the participants and investigators was ensured by treating the women with identically appearing tablets of aspirin or placebo (Bayer AG, Leverkusen, Germany).
Table 7. Summary of the material and methods and the pregnancy outcome data of the studies.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Study I n=374</th>
<th>Study II n=122</th>
<th>Study III n=72</th>
<th>Study IV n=176</th>
<th>Study V n=487</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study Outcome measures</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>PR/cycle</td>
<td>UTA PI</td>
<td>Maternal serum placental proteome profile during the first half of pregnancy</td>
<td>UTA PI</td>
<td>Hypertensive pregnancy complications</td>
</tr>
<tr>
<td>Secondary</td>
<td>Number of oocytes, number and quality of embryos, LBR, miscarriage and extraterine rate, number of top-quality embryos, PR/ET, PR/eSET</td>
<td>Bilateral UTA PI ≥ 3.0, arcuate radial and spiral artery PI, endometrial thickness</td>
<td>Bilateral UTA notch, arcuate and umbilical, artery PI</td>
<td>IUGR, inter-twin growth discordance, bleeding during pregnancy, blood loss at delivery</td>
<td></td>
</tr>
<tr>
<td>Treatment group</td>
<td>ASA</td>
<td>Placebo</td>
<td>ASA</td>
<td>Placebo</td>
<td>ASA</td>
</tr>
<tr>
<td>Randomized Subjects</td>
<td>186</td>
<td>188</td>
<td>61</td>
<td>61</td>
<td>Subgroup from previous studies</td>
</tr>
<tr>
<td>Withdrew after giving informed consent (ET n (%))</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>PR/ET n (%)</td>
<td>174 (93.5)</td>
<td>175 (93.1)</td>
<td>57 (93.4)</td>
<td>56 (91.8)</td>
<td>80 (92.0)</td>
</tr>
<tr>
<td>LB/ET n (%)</td>
<td>44 (25.3)</td>
<td>48 (27.4)</td>
<td>15 (26.3)</td>
<td>16 (28.6)</td>
<td>18 (22.5)</td>
</tr>
<tr>
<td>Miscarriage/PR n (%)</td>
<td>32 (18.4)</td>
<td>37 (21.1)</td>
<td>13 (22.8)</td>
<td>13 (23.2)</td>
<td>15 (18.8)</td>
</tr>
<tr>
<td>Extraterine pregnancy/PR n (%)</td>
<td>8 /18.2</td>
<td>8 (16.7)</td>
<td>2 (13.3)</td>
<td>2 (12.5)</td>
<td>All patients had uncomplicated pregnancy and labour</td>
</tr>
<tr>
<td>Analysed subjects</td>
<td>174</td>
<td>175</td>
<td>57</td>
<td>56</td>
<td>15</td>
</tr>
</tbody>
</table>

ET=embryo transfer, PR=clinical pregnancy rate, LB/(R)=live birth (rate), eSET=elective single embryo transfer, UTA=uterine artery PI=pulsatility index, IUGR=intrauterine growth restriction.
4.3 Stimulation protocol

A long gonadotrophin-releasing hormone (GnRH) agonist protocol was carried out, and buserelin (Suprecur®; Hoechst AG, Frankfurt, Germany) or nafarelin (Synarel®; Syntex Nordica AB, Södertälje, Sweden) were started for down-regulation on cycle days 20–22. When ovarian suppression was confirmed ultrasonographically, recombinant follicle-stimulating hormone (FSH) (Gonal-F®, Laboratories Serono; or Puregon®, Organon, Oss, the Netherlands) or human menopausal gonadotrophin (hMG) (Menogon®, Ferring, the Netherlands) were used for controlled ovarian hyperstimulation. Each clinic used their routine treatment regimens. Oocyte retrieval was performed 34–36 hours after injection of 5000–10 000 IU of human chorionic gonadotrophin (Profasi®, Ares-Serono; or Pregnyl®, Organon). Fertilized oocytes were cultured in MediCult Medium® (Medi-Cult A/S, Copenhagen, Denmark), IVF-500 Medium® (Scandinavian IVF Science, Gothenburg, Sweden) or Sydney IVF Medium® (Cook IVF, Queensland, Australia). One top-quality embryo was electively transferred into the uterine cavity 46–50 h after oocyte retrieval in subjects under 39 years and undergoing their first or second stimulation. Otherwise, two embryos were transferred. The criteria for a top-quality embryo were: normal fertilization (2PN), 4 or 5 evenly sized blastomeres on day 2 or ≥8 on day 3, less than 20% fragmentation and no multinuclear blastomeres (Van Royen et al. 1999). Natural progesterone (Lugesterone®, Leiras, Finland) was given transvaginally (200 mg × 3) for luteal support for 14 days. Clinical pregnancies were confirmed by transvaginal ultrasonography five weeks after ET.

4.4 Ultrasonographic examinations (II, IV)

Ultrasonographic examinations (equipment: Acuson Sequoia 512; Mountain View, CA, USA) were performed on the day of embryo transfer and four times during the first half of pregnancy (6+, 10+, 13+ and 18+ gestational weeks). The insonation angle was minimized to <30° in every measurement and the high pass filter was set at its minimum. A continuous waveform of at least three consecutive cardiac cycles was required for successful examination. All the ultrasonographic data were videotaped and analysed afterwards, using the ultrasound equipment’s own cardiac measurement package.
4.4.1 Embryo transfer day

A 5–8 MHz transvaginal transducer (EV-8C4) was used. All women were examined before noon. Both UtAs were located by colour Doppler ultrasonography and their blood flow velocity waveforms were obtained at the cervicocorporeal level of the uterus. Arcuate arteries were identified bilaterally and blood velocity waveforms were obtained as distally as possible from the uterine artery. Radial artery blood velocity waveforms were obtained from the middle of the myometrial region. A colour Doppler window was then placed over the thickest part of the endometrium. The highest colour intensity from the endometrial/subendometrial area was located and its blood velocity waveforms were obtained. This vessel represented the spiral artery (Schild et al. 2001). The thickness of the endometrium was measured as the maximum distance between the myometrial/endometrial interface through the central longitudinal axis of the uterus (Zaidi et al. 1995). Three consecutive measurements were obtained and their mean value was used for further analysis.

Pulsatility index (PI = (peak systolic velocity – end-diastolic velocity) / time-averaged maximum velocity over the cardiac cycle) values were calculated from three consecutive cardiac cycles and their mean value was used for further analysis. The side with the lowest PI value was chosen for final analysis. The incidence of a bilateral UtA PI value of ≥3.0 (non-optimal uterine haemodynamics), which is considered to predict poor outcome in IVF and ICSI (Steer et al. 1992a, Zaidi et al. 1996), was determined. To calculate intra-observer variability of the UtA PI measurements, UtA blood flow velocity waveforms were obtained twice, 10–15 minutes apart from ten consecutive patients.

4.4.2 Early and mid-pregnancy

At 6+ and 10+ weeks of gestation, examinations were performed transvaginally using an 8-MHz transducer (EV-8C4), and at 13+ and 18+ gestational weeks transabdominally using an 8-MHz convex transducer.

Gestational age was determined in relation to the day of ET. Normal fetal growth and development was confirmed during each ultrasonographic examination. At 13+ gestational weeks nuchal translucency was measured and at 18+ weeks of gestation a detailed fetal anatomic examination was performed.

Image-directed pulsed and colour Doppler ultrasonography was used to obtain blood velocity waveforms from both (proximal) UtAs at every examination. In addition, at 6+ and 10+ gestational weeks subplacental arcuate arteries were
identified and their blood velocity waveforms were obtained. Pulsatility index values were calculated. Early diastolic notches of UtAs were noted. At 10+, 13+ and 18+ gestational weeks umbilical artery blood flow velocity waveforms were obtained near the abdominal insertion of the umbilical cord. Umbilical artery PI values and mean velocity (Vmean = fetal heart rate × time-velocity integral) were calculated. Fetal heart rate was measured from umbilical artery blood velocity waveforms.

The acoustic output of the system was displayed via mechanical and thermal indices, which were kept at <1.0 (Morin et al. 2005).

4.5 Pregnancy and delivery data (I, II, IV, V)

Pregnancy and delivery data were collected from hospital registers. Miscarriage was defined as pregnancy loss before 12 full weeks of gestation. Pregnancy-induced hypertension and pre-eclampsia were classified according to ACOG criteria (ACOG 2002). IUGR was defined as a birth-weight below the fifth centile according to Finnish fetal growth curves (Pihkala et al. 1989). Prematurity was defined as delivery before 37 full gestational weeks. Inter-twin growth discordance of >20% was considered significant.

4.6 Proteomic analysis (III)

4.6.1 Sample collection

For early and mid-pregnancy control groups, we identified healthy women with no risk factors of hypertensive pregnancy complications from outpatient maternity clinics for blood sampling. Those women who experienced uncomplicated pregnancy after spontaneous conception and normal vaginal delivery were chosen as control subjects. All blood samples were allowed to clot for 30 min, centrifuged at 3000 × g and the supernatants were collected and stored at -80 °C until further processing.

4.6.2 Analysis

Fluorescence Two-Dimensional Differential in-Gel Electrophoresis (2D-DIGE)

To improve the detection of low-abundance proteins, serum samples were depleted of six major proteins by using a Multiple Affinity Removal System (Agilent Technologies, Inc., Palo Alto, CA). For proteomic analysis serum samples from 10
women with spontaneous pregnancy and 10 placebo-treated IVF/ICSI patients were pooled for first trimester (10–12 gestational weeks) samples, and samples from 10 placebo- and 10 aspirin-treated IVF/ICSI patients were pooled to create samples for two gestational age ranges (10–12 and 18–22 gestational weeks) to run in 2D DIGE. Serum proteins (50 µg) were labelled with CyDye DIGE Fluor minimal dye (GE Healthcare Bio-Sciences, Piscataway, NJ) at a concentration of 100–400 pmol of dye/50 µg of protein. Samples were labelled with Cy3 (Spontaneous pregnancy or Aspirin IVF/ICSI), Cy5 (Placebo IVF/ICSI), or Cy2 (reference, Spontaneous pregnancy + Placebo IVF/ICSI; reference, Placebo IVF/ICSI + Aspirin IVF/ICSI), multiplexed and resolved in one gel. Labelled proteins were purified by acetone precipitation, dissolved in IEF buffer, and rehydrated on a 24- or 13-cm immobilized pH gradient (IPG) strip (pH 4–7) for 12 h at room temperature. The IPG strip was subjected to first-dimension electrophoresis at 65–70 kVh and then equilibrated with dithiothreitol and isoamyl alcohol equilibration buffers for 15 min sequentially. Second-dimension electrophoresis was conducted at 80–90 V for 18 h. Gels were scanned in a Typhoon 9400 scanner (GE).

**Multidimensional Liquid Chromatography Tandem Mass Spectrometry (LC–MS/MS) and MALDI–TOF–MS**

For protein identification the spots from 2D preparative gels were digested with trypsin, and samples representing each group were analysed on a Q-Tof-2 mass analyser connected to capillary LC (CapLC, Waters) and MALDI-TOF-MS equipment, with a pulsed-ion extraction source. Masses from m/z 400 to 1500 were scanned for MS survey, and masses from m/z 50 to 199 were scanned for MS/MS. Data analysis was performed using ProteinLynx Global Server v.2.1 (Waters) and *de novo* sequencing using a PEAKS algorithm combined with the OpenSea alignment algorithm (v.1.3.1). The Swiss-Prot database was used to identify proteins and peptides of five or more amino acids. Proteins with two or more peptides according to ProteinLynx (significance score >10.6) were chosen. For MALDI-TOF, collected spectra were analysed against the Swiss-Prot database using Mascot software, and scores above 53 (p-value <0.05) were considered positive matches.

**4.6.3 Enzyme-linked immunosorbent assay**

Concentrations of 11 biomarker proteins in spontaneous pregnancies, placebo- and aspirin-treated IVF/ICSI patients’ serum samples were determined by enzyme-
linked immunosorbent assays (ELISAs) (Engvall & Perlman 1971). Specific antibodies and pure proteins (for standards) were obtained from multiple vendors (Dako, RND or Academy biomed) for the sandwich ELISAs. The concentrations of individual proteins were estimated from the average values of triplicates in comparison with the standard curves.

4.7 Outcome measures

Primary and secondary outcome measures of the studies are listed in Table 7. The primary outcome measure of Study I was clinical pregnancy rate per cycle. In the ultrasonographic studies (II, IV) primary outcome variables were UtA PI values on the day of ET and at 18+ gestational weeks. In Study III the main interest was placental proteomic profile differences in spontaneous and placebo- or aspirin-treated IVF and ICSI pregnancies. Study V was a follow-up study of a total of 487 IVF and ICSI patients and the main outcome measure was the incidence of hypertensive pregnancy complications.

4.8 Sample size and statistical analysis

Sample sizes for Studies I, II, IV and V were calculated by using Stata Statistics/Data Analysis 8.0 (Stata Corporation, College Station, Texas, USA). All calculations were based on primary outcome measures (Table 7).

4.8.1 Sample size

Study I

In Study I, sample size calculation was targeted at proving a 15% increase in clinical pregnancy rate per cycle in favour of the aspirin group. Based on the calculation with $\alpha = 0.05$ and a power of 80%, 176 subjects were needed in each group. The expected clinical pregnancy rate per cycle initiated in the control group was 27% based on previous pregnancy rate results in the Infertility Clinic of Oulu University Hospital.

Study II

Sample size was calculated so as to be able to detect a decrease in UtA PI values from 3.0 (Steer et al. 1992a, Zaidi et al. 1996) to 2.6 (Salle et al. 1998), using an SD value
of 0.5 on the day of ET in favour of the aspirin group. To detect such a decrease with \( \alpha \)-error of 0.05 and a statistical power of 90\%, we needed 49 subjects in each group.

**Study III**

The superior sensitivity of mass spectrometry compared with other spectroscopic methods is its main asset. Thus, for proteomic approaches only five to ten samples representing each group enable reliable identification and quantification of differentially expressed proteins between the pooled groups (Nilsson 1994). Validation of the proteins by ELISA was carried out individually.

**Study IV**

Sample size was calculated to detect a decrease in uterine artery PI value from 1.5 (Papageorghiou et al. 2001, Albeiges et al. 2003) to 1.0 at 18 + gestational weeks in favour of the aspirin group, with \( \alpha = 0.05 \) and a power of 80\%. Based on the calculation, 16 subjects were needed in each group.

**Study V**

Sample size calculation was based on the results of a previous randomized, placebo-controlled and double-blind study among IVF and ICSI women by Lambers et al. (Lambers et al. 2009b). In their study, the incidence of hypertensive pregnancy complications was statistically significantly lower in the aspirin group (3.6\%, \( n = 28 \)) than in the placebo group (26.9\%, \( n = 26 \)). To show a similar (23\%) decrease in the incidence of hypertensive pregnancy complications in favour of the aspirin group, with \( \alpha \)-error of 0.05 and a power of 80\%, 46 subjects were needed in each group.

### 4.8.2 Statistical analysis

For Studies I, II, IV and V statistical analyses were performed with SPSS software, versions 10.1–16.0 (SPSS Inc., Chicago, IL, USA). Comparison of quantitative normally distributed data between the groups was carried out by using the two-tailed \( t \)-test; otherwise the Mann–Whitney \( U \) test was chosen. Comparison of categorial data between groups was carried out by using Pearson’s \( \chi^2 \) test or Fisher’s exact test for variables with low frequencies (i.e. >20\% of expected frequencies are less than 5 in a \( 2 \times 2 \) table). Comparison of measured parameters within a group at
multiple study points was performed by one-way analysis of variance (ANOVA) for repeated measurements (Study IV). Confidence intervals (95%) were calculated for the difference in the proportion of bilateral UtA PI values of ≥3.0 (II) and for hypertensive pregnancy complications (V) between the study groups. An intention-to-treat approach was used in comparisons of characteristic and IVF/ICSI protocol parameters. A *p* value <0.05 was considered statistically significant.

In Study III spectral counts from proteins identified in 2D-LC-MS/MS were subjected to independent pair-wise comparisons to determine differentially expressed proteins in spontaneous pregnancies and placebo- or aspirin-treated IVF/ICSI groups, as well as between placebo and aspirin-treated IVF/ICSI patients. Pair-wise comparisons were performed using either a 2 × 2 chi-square test or Fisher’s exact test for lower abundance proteins. Normalization of spectral counts to account for experimental variability was built into the pair-wise comparisons. The fold expression change of differentially expressed proteins between groups was quantified by using a previously published equation (Old *et al.* 2005). The method was automated using an SAS program (version 9.1) and all proteins were independently tested. The level of significance was set at 0.05.

Candidate protein biomarker concentrations (expressed as ng/mL) measured by ELISA were log-transformed before subjecting them to statistical analysis. Subjects with adequate overall protein in their samples, but with ELISA values under the detection limit for a particular protein were assigned a value of 0.1 rather than 0 to facilitate log-transformation. When transformed to a log scale, the value of -2.3 corresponded to those with no protein detected. Independent pair-wise comparisons of log-transformed protein concentrations between spontaneous pregnancies and placebo-treated IVF/ICSI patients, between spontaneous pregnancies and aspirin-treated IVF/ICSI patients, as well as between placebo and aspirin-treated IVF/ICSI patients were performed by using ANOVA. Comparisons of the log-transformed protein concentrations within groups at multiple study points were performed by one-way ANOVA for repeated measurements. Average values on the log-scale were transformed back to original units (harmonic mean) for presentation. Bonferroni correction was applied to adjust for multiple comparisons. The descriptive and comparative analyses were conducted using SAS software (v9.1).
5 Results

Low-dose aspirin and clinical parameters in IVF and ICSI patients

Clinical characteristics are shown in Table 8. Male infertility was more common \((p < 0.05)\) in the placebo group (45%) than in the aspirin group (12%) in Study III. Otherwise, there were no statistically significant differences in maternal, perinatal or neonatal parameters between the study groups in Studies I, II, IV and V.

5.1.1 Treatment and pregnancy outcome (I, V)

In Study I the use of recombinant FSH or hMG was similar in the aspirin and placebo groups. No significant differences were observed in mode of treatment (IVF, ICSI or combined techniques) between the groups. Neither were there significant differences in ovarian responsiveness (number and quality of oocytes), fertilization rate, number of top-quality embryos transferred or total number of top-quality embryos between the groups. The outcomes of treatment in both groups are presented in Tables 9a and 9b.

In Studies I, II, IV and V embryo transfer was possible in 92–93.8% of cases in the aspirin group and in 91.8–93.5% in the placebo group (Table 7). In Study I, elective single ET and double ET rates were comparable in the aspirin and placebo groups. The incidence of women who did not undergo ET was similar in both groups. In these cases ET could not be performed because of a poor response to gonadotrophin treatment, unsuccessful fertilization, premature ovulation or a low blastocyst cleavage rate.

The clinical pregnancy rate (per cycle and per ET) and live-birth rate did not differ statistically significantly between aspirin- and placebo-treated subjects, as shown in Table 9a. By increasing the sample size (Study V), pregnancy rate and live-birth rate slightly increased, whereas adverse early pregnancy outcomes decreased in both groups. In Study I, the clinical pregnancy rate per ET was also analysed separately for each of four clinics (University Hospitals of Oulu, Kuopio and Tampere, and The Family Federation Infertility Clinic, Oulu) and the results did not differ statistically significantly between the centres as regards the aspirin (17.6–37.5%) and the placebo (20.8–29.6%) groups. When the subjects were divided into two groups according to maternal age (< 35 years and ≥ 35 years) and cycle number (1st vs. 2nd or 3rd), no statistically significant differences were found in variables of ovarian responsiveness or in pregnancy rate between the study groups.
Table 8. Clinical characteristics.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Study I</th>
<th>Study II</th>
<th>Study IV</th>
<th>Study V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ASA (n=186)</td>
<td>Placebo (n=188)</td>
<td>ASA (n=61)</td>
<td>Placebo (n=61)</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>32 (24-39)</td>
<td>31 (22-39)</td>
<td>32 (24-39)</td>
<td>31 (22-39)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.0 (17.3-37.3)</td>
<td>24.7 (18.1-38.9)</td>
<td>24 (18-37)</td>
<td>24 (19-38)</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>3 (1-11)</td>
<td>4 (1-14)</td>
<td>3.8 (1.9)</td>
<td>4.1 (2.5)</td>
</tr>
<tr>
<td>Aetiology of infertility (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>28</td>
<td>29</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>Tubal</td>
<td>13</td>
<td>15</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>22</td>
<td>19</td>
<td>31</td>
<td>28</td>
</tr>
<tr>
<td>Hormonal</td>
<td>9</td>
<td>11</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Unexplained</td>
<td>23</td>
<td>19</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Mixed</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Pregnancy history (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous pregnancy</td>
<td>34</td>
<td>38</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td>Live birth</td>
<td>49</td>
<td>57</td>
<td>61</td>
<td>62</td>
</tr>
</tbody>
</table>

*p<0.05. Data are given as medians (range) (I-II) or means (±SD) (IV-V).
5.1.2 Hypertensive pregnancy complications (V)

The overall incidence of hypertensive pregnancy complications did not differ statistically significantly between the aspirin and the placebo groups. The incidences of both mild and severe pre-eclampsia were comparable in the study groups. In addition, there were no statistically significant differences in other perinatal or neonatal parameters (Table 9b).

Table 9. a) Outcome of IVF/ICSI and fresh embryo transfer in the study groups (I and V).

<table>
<thead>
<tr>
<th>Variable</th>
<th>ASA (n=186/242)</th>
<th>Placebo (n=188/245)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian responsiveness (Study I)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oocytes</td>
<td>12.0±7.0</td>
<td>12.7±7.2</td>
</tr>
<tr>
<td>Fertilized</td>
<td>6.5±4.5</td>
<td>6.8±5.9</td>
</tr>
<tr>
<td>Cleaved</td>
<td>5.8±4.3</td>
<td>6.0±4.7</td>
</tr>
<tr>
<td>Frozen</td>
<td>1.3±2.4</td>
<td>1.5±2.5</td>
</tr>
<tr>
<td>No. of embryos</td>
<td>5.8±4.35</td>
<td>5.99±4.66</td>
</tr>
<tr>
<td>No. of top-quality embryos</td>
<td>0.99±1.39</td>
<td>1.18±1.51</td>
</tr>
<tr>
<td>No. of embryos transferred</td>
<td>1.64±0.64</td>
<td>1.63±0.71</td>
</tr>
<tr>
<td>No. of top-quality embryos transferred</td>
<td>0.57±0.65</td>
<td>0.62±0.62</td>
</tr>
<tr>
<td>PR/eSET (%)</td>
<td>27.5</td>
<td>30.9</td>
</tr>
<tr>
<td>Outcome of treatment (Studies I, V)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR/cycles initiated (%)</td>
<td>23.7/26.4</td>
<td>25.5/27.3</td>
</tr>
<tr>
<td>PR/ET (%)</td>
<td>25.3/28.2</td>
<td>27.4/29.3</td>
</tr>
<tr>
<td>Pregnancy outcome (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live birth/ET</td>
<td>18.4/22.9</td>
<td>21.1/24.0</td>
</tr>
<tr>
<td>Miscarriage/PR</td>
<td>18.2/12.5</td>
<td>16.7/13.4</td>
</tr>
<tr>
<td>Extrauterine pregnancy/PR</td>
<td>9.0/6.3</td>
<td>6.3/4.5</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD. n=Study I/Study V, PR=clinical pregnancy rate, eSET=elective single embryo transfer, ET=embryo transfer
### Table 9. b) Pregnancy outcomes and complications in the aspirin and placebo groups (V).

<table>
<thead>
<tr>
<th>Variable</th>
<th>ASA (n=52)</th>
<th>Placebo (n=55)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertensive complication n (%)</td>
<td>8 (15.4)</td>
<td>10 (18.2)</td>
<td>0.70</td>
</tr>
<tr>
<td>PIH</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Pre-eclampsia</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>IUGR in singletons n (%)</td>
<td>2/41 (4.9)</td>
<td>3/40 (7.5)</td>
<td>0.68</td>
</tr>
<tr>
<td>Vaginal bleeding during pregnancy n (%)</td>
<td>2 (3.8)</td>
<td>2 (3.6)</td>
<td>1.00</td>
</tr>
<tr>
<td>GA at delivery (weeks)</td>
<td>38 (31-42)</td>
<td>38 (28-41)</td>
<td>0.85</td>
</tr>
<tr>
<td>Mode of delivery n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caesarean section</td>
<td>16 (31)</td>
<td>15 (27)</td>
<td>0.83</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singletons</td>
<td>3446 (465)</td>
<td>3380 (645)</td>
<td>0.60</td>
</tr>
<tr>
<td>Twin A</td>
<td>2440 (410)</td>
<td>2560 (857)</td>
<td>0.65</td>
</tr>
<tr>
<td>Twin B</td>
<td>2180 (648)</td>
<td>2515 (875)</td>
<td>0.29</td>
</tr>
<tr>
<td>Apgar score at 5 min*</td>
<td>9 (7-10)</td>
<td>9 (6-10)</td>
<td>0.51</td>
</tr>
<tr>
<td>Umbilical cord blood pH</td>
<td>7.25 (0.06)</td>
<td>7.24 (0.07)</td>
<td>0.98</td>
</tr>
<tr>
<td>Blood loss at delivery (ml)</td>
<td>682 (478)</td>
<td>633 (499)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Data are given as mean (+SD) or median (range)*. *4 twin pregnancies in the aspirin group and 5 twin pregnancies in the placebo group. *1 singleton and 1 twin pregnancy in the aspirin group, 1 singleton and 2 twin pregnancies in the placebo group, *analysed by Fisher’s exact test.

5.2 Effects of low-dose aspirin on uterine and placental haemodynamics (II, IV)

#### 5.2.1 Uterine receptivity

Mean uterine artery PI values did not differ significantly between the aspirin and the placebo groups. The incidence of non-optimal uterine haemodynamics (bilateral UtA PI values ≥ 3.0) was more frequent in the placebo group than in the aspirin group. None of the women with non-optimal uterine haemodynamics had clinical pregnancy. Arcuate, radial and spiral artery PI values and endometrial thickness did not differ significantly between the groups. The number of retrieved oocytes and the number of top-quality embryos were comparable between the women with non-optimal (bilateral UtA PI ≥3.0) and optimal (UtA PI <3.0) uterine haemodynamics. In addition, there was no statistically significant difference (p = 0.46) in mean PI values in the UtA between women who conceived after ET and
those who did not. The data on parameters representing uterine receptivity on the ET day is shown in Table 10.

5.2.2 Uterine and placental haemodynamics in early and mid-pregnancy

In Study IV, the study population in the first ultrasonographic examination (6+ weeks’ gestation) consisted of 37 subjects (17 aspirin and 20 placebo) as a result of early miscarriages. In the aspirin group one patient had first-trimester miscarriage (between 6+ and 10+ gestational weeks) and one patient discontinued the study medication because of early pregnancy bleeding. These patients were included in the final data analysis. Thirty-five subjects, of whom 15 received aspirin and 20 placebo, underwent the complete ultrasonographic protocol. There were 4 dichorionic-diamniotic twin pregnancies in the aspirin group and 3 in the placebo group.

At 6+ gestational weeks, arcuate artery PI values were lower in the aspirin group compared with the placebo group (Figure 4, Table 10), while UtA PI values did not differ significantly between the groups (Figure 5, Table 10).

Uterine artery PI values decreased with advancing gestation in both groups. At 18+ gestational weeks, PI values of the right and left UtAs were significantly lower in the aspirin group than in the placebo group (Figure 5, Table 10). Bilateral early diastolic notches in the UtAs were noted in every case at 6+ gestational weeks. With advancing gestation, the incidence of bilateral UtA notches decreased in both groups. At 18+ gestational weeks, bilateral notches tended to be more common in the placebo group than in the aspirin group (Table 10). The results were similar in a subgroup analysis in which all the twin pregnancies were excluded.

Umbilical artery PI values decreased and Vmean increased significantly with advancing gestation in both groups, with no statistically significant differences between the groups (Table 10).
Fig. 4. Arcuate artery pulsatility index values at 6 and 10 weeks' gestation (mean ± SD). *p<0.05 compared with placebo group.

Fig. 5. Right and left uterine artery pulsatility index values at 6, 10, 13 and 18 weeks' gestation (mean ± SD). *p<0.05 compared with placebo group.
Table 10. Haemodynamic parameters in the aspirin and placebo groups (II, IV).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Study II</th>
<th>Study IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ET-day</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ASAs</td>
<td>Placebos</td>
</tr>
<tr>
<td></td>
<td>n=57</td>
<td>n=56</td>
</tr>
<tr>
<td>Arcuate artery PI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6+ weeks’ gestation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA</td>
<td>2.02</td>
<td>1.98</td>
</tr>
<tr>
<td>(1.21-3.36)</td>
<td></td>
<td>(1.15-3.10)</td>
</tr>
<tr>
<td>Placebo</td>
<td>1.71 (0.45)*</td>
<td>2.34 (0.61)</td>
</tr>
<tr>
<td>10+ weeks’ gestation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA</td>
<td>2.35</td>
<td>2.51</td>
</tr>
<tr>
<td>(1.23-3.76)</td>
<td></td>
<td>(1.17-3.82)</td>
</tr>
<tr>
<td>Placebo</td>
<td>1.95 (0.45)*</td>
<td>2.60 (0.62)</td>
</tr>
<tr>
<td>13+ weeks’ gestation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA</td>
<td>2.59</td>
<td>2.35</td>
</tr>
<tr>
<td>(0.63-0.83)</td>
<td></td>
<td>(0.73-0.78)</td>
</tr>
<tr>
<td>Placebo</td>
<td>2.38 (0.67)</td>
<td>2.88 (0.74)</td>
</tr>
<tr>
<td>18+ weeks’ gestation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA</td>
<td>2.67</td>
<td>2.56</td>
</tr>
<tr>
<td>(0.31-0.61)</td>
<td></td>
<td>(0.53-0.61)</td>
</tr>
<tr>
<td>Placebo</td>
<td>2.62 (0.45)*</td>
<td>3.03 (0.60)</td>
</tr>
<tr>
<td>Bilateral PI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 3.0 n (%)</td>
<td>5 (8.8)*</td>
<td>13 (23.2)</td>
</tr>
<tr>
<td>Bilateral notch n (%)</td>
<td>17 (100)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Umbilical artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>2.87 (0.31)</td>
<td>3.03 (0.25)</td>
</tr>
<tr>
<td>Vmean (cm/s)</td>
<td>5.76 (1.71)</td>
<td>5.63 (1.46)</td>
</tr>
<tr>
<td>Endometrial thickness (mm)</td>
<td>9.9</td>
<td>9.4</td>
</tr>
<tr>
<td>(7.2-13.0)</td>
<td>(6.9-14.1)</td>
<td></td>
</tr>
</tbody>
</table>

Data are given as medians (95% CI) in Study II and as means (±SD) in Study IV. ET=embryo transfer, n=mothers/fetuses, PI=pulsatility index, Vmean=mean velocity.

*p<0.05 vs. placebo group, **p=0.06, ±95% CI for proportions 1.4 to 27.9%.
5.3 Proteomic analysis in early pregnancy (III)

The demographic and clinical characteristics of the study groups are presented in Table 11. Maternal age was significantly higher in placebo- \((p<0.001)\) and aspirin- \((p=0.01)\) treated IVF/ICSI patients compared with spontaneous pregnancies. Between IVF/ICSI groups maternal age did not differ significantly. There were no statistically significant differences in characteristic variables or fertilization parameters between placebo- and aspirin-treated IVF/ICSI women.

Table 11. Demographic and clinical characteristics of the study groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Spontaneous pregnancy ((n=42))</th>
<th>Placebo IVF/ICSI ((n=15))</th>
<th>Aspirin IVF/ICSI ((n=15))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>26.4 (4.0)</td>
<td>31.5 (3.9)*</td>
<td>30.0 (2.8)*</td>
</tr>
<tr>
<td>Smoking % (n)</td>
<td>2.4 (1)</td>
<td>0</td>
<td>6.7 (1)</td>
</tr>
<tr>
<td>Nulliparity % (n)</td>
<td>73.8 (30)</td>
<td>80.0 (12)</td>
<td>86.6 (13)</td>
</tr>
<tr>
<td>GA at enrolment (weeks)</td>
<td>10.7 (0.4)</td>
<td>10.9 (0.2)</td>
<td>10.5 (0.2)</td>
</tr>
<tr>
<td>GA at delivery (weeks)</td>
<td>39.4 (1.18)</td>
<td>39.0 (3.0)</td>
<td>38.5 (2.2)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3520 (410)</td>
<td>3380 (655)</td>
<td>3300 (515)</td>
</tr>
<tr>
<td>Apgar score at 5 min*</td>
<td>9 (5-10)</td>
<td>9 (6-10)</td>
<td>9 (7-10)</td>
</tr>
<tr>
<td>Umbilical artery pH</td>
<td>7.22 (0.11)</td>
<td>7.23 (0.07)</td>
<td>7.23 (0.08)</td>
</tr>
</tbody>
</table>

Data are shown as means \((\pm SD)\) or medians (range)*. GA=gestational age, *Comparison between controls and placebo and aspirin IVF/ICSI, \(p<0.02\).

5.3.1 Longitudinal changes of maternal serum placental proteins during the first half of pregnancy

In each group, maternal serum concentrations of chorionic somatomammoprotein 1 (CSH1) and pappalysin 1 (PAPP-A1) increased and the concentration of beta human chorionic gonadotrophin (β-hCG) decreased statistically significantly between 10–12 and 18–22 gestational weeks. The concentrations of pregnancy-specific β1 glycoprotein 1 (PSG), lipopolysaccharide binding protein (LBP) and C-reactive protein (CRP) increased significantly only in the control group. Maternal serum vasorin concentrations decreased statistically significantly both in the spontaneous pregnancies and in the placebo IVF/ICSI groups. In addition, in the placebo IVF/ICSI group, the concentration of complement factor D (CFD) decreased statistically significantly between 10–12 and 18–22 gestational weeks.
5.3.2 IVF/ICSI pregnancy versus spontaneous pregnancy

Pseudo-colour visualization of protein patterns, with spontaneous pregnancy spots in green and placebo IVF/ICSI spots in red, revealed distinct patterns of differentially expressed proteins in the spontaneous pregnancies and placebo IVF/ICSI groups at 10–12 weeks of gestation (Figures 6 and 7). Mass spectrometry analysis allowed us to identify a total of 368 unique proteins. Potential serum biomarkers showing significant differences included extra-cellular matrix proteins, cytoskeletal, vascular, complement and transport proteins.

Fig. 6. 2D-DIGE gel at 10–12 gestational weeks with placebo IVF/ICSI and spontaneous pregnancy groups overlaid (A). Spots which fluoresce as yellow represent proteins which are equally present in both samples and are essentially non-informative. Red spots represent proteins that are over-expressed in the IVF/ICSI sample and green spots represent proteins that are over-expressed in the spontaneous pregnancy sample. Figure B displays a difference map generated by using Phoretix 2D Evolution software showing >2-fold differentially expressed spots. Up-regulated spots in spontaneous pregnancies (green) compared with down-regulated spots in placebo-treated IVF/ICSI women (red).
Fig 7. The upper panel displays a difference map generated by using Phoretix 2D gel Evolution software (see Fig. 6b) stained with Coomassie blue. The lower panel displays an LC-MS/MS spectrum from spot 5, showing tryptic peptide.

As shown in Table 13, concentrations of PSG1, CSH1 and LBP were statistically significantly higher in the placebo-treated IVF/ICSI group at 10–12 gestational weeks compared with spontaneous pregnancies. At 18–22 gestational weeks only PSG1 remained at higher concentrations at the level of statistical significance.
5.3.3 Low-dose aspirin versus placebo in IVF pregnancies

Pseudo-colour visualization of protein patterns, with aspirin spots in green and placebo spots in red, showed a unique and distinct pattern of differentially expressed proteins in the aspirin- and placebo-treated IVF/ICSI subjects (Figure 8). The detection protocol allowed us to identify >800 spots from each gel set. Gel quantification showed differences in relative abundance in >40 spots (>2 fold, \( p < 0.05 \)). Protein identification of 176 spots by MALDI-TOF-MS and LC-MS/MS revealed 62 unique proteins in the samples. Thirty-two of these proteins, selected on the basis of magnitude of difference, are listed in Table 12. Differentially expressed maternal serum placental proteins included extra-cellular matrix proteins, complement proteins and transport proteins. However, maternal serum concentrations of \( \beta \)-hCG, PSG1, CSH1, LBP, PAPP-A1, CRP, vascular cell adhesion molecule 1 (VCAM1), fibronectin, endoglin, vasorin and CFD did not differ statistically significantly between the groups at 10–12 or at 18–22 gestational weeks (Table 13).

![Fig 8. 2D DIGE images of pooled aspirin group (green) versus pooled placebo group (red) maternal serum (IVF/ICSI subjects) at 10–12 gestational weeks (A), and at 18–22 gestational weeks (B). Green spots represent proteins that are over-expressed in aspirin-treated IVF/ICSI patients and red spots represent proteins that are under-expressed in placebo-treated IVF/ICSI patients.](image-url)
Table 12. Proteins identified by using 2D-DIGE, followed by MALDI-TOF-MS and LC-MS-MS.

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Protein symbol</th>
<th>Protein ID</th>
<th>Fold change Aspirin vs. Placebo (18-22 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>141</td>
<td>AACT_HUMAN</td>
<td>Alpha-1-antichymotrypsin precursor</td>
<td>&gt;10.0</td>
</tr>
<tr>
<td>133</td>
<td>PHL1_HUMAN</td>
<td>Phosphatidylinositol-glycan-specific phospholipase D1 precursor</td>
<td>&gt;10.0</td>
</tr>
<tr>
<td>59, 60, 61, 62, 63</td>
<td>A2GL_HUMAN</td>
<td>Leucine-rich-alpha-2-glycoprotein precursor</td>
<td>11.3</td>
</tr>
<tr>
<td>224, 225, 226</td>
<td>CFAH_HUMAN</td>
<td>Complement factor H precursor</td>
<td>7.9</td>
</tr>
<tr>
<td>98, 159, 160, 161, 162, 183, 185, 198, 199, 200, 201, 202, 203, 204, 205, 206</td>
<td>CERU_HUMAN</td>
<td>Ceruloplasmin precursor (Ferroxidase)</td>
<td>7.7</td>
</tr>
<tr>
<td>157, 158, 163, 166, 167, 168, 169</td>
<td>A1BG_HUMAN</td>
<td>Alpha-1B-glycoprotein precursor</td>
<td>3.0</td>
</tr>
<tr>
<td>65, 66, 67, 68, 69</td>
<td>ZA2G_HUMAN</td>
<td>Zinc-alpha-2-glycoprotein precursor</td>
<td>2.5</td>
</tr>
<tr>
<td>106, 109, 111, 112, 113, 150, 170, 172, 173, 174, 175, 176, 177, 178, 179</td>
<td>HEMO_HUMAN</td>
<td>Hemopexin precursor (Beta-1-B-glycoprotein)</td>
<td>2.2</td>
</tr>
<tr>
<td>1, 2, 15, 16</td>
<td>THRB_HUMAN</td>
<td>Prothrombin precursor</td>
<td>2.2</td>
</tr>
<tr>
<td>70, 71, 72, 73, 74, 75, 124, 139</td>
<td>IC1_HUMAN</td>
<td>Plasma protease C1 inhibitor precursor</td>
<td>2.1</td>
</tr>
<tr>
<td>23</td>
<td>CO4_HUMAN</td>
<td>Complement C4 precursor</td>
<td>1.6</td>
</tr>
<tr>
<td>220</td>
<td>VP28_HUMAN</td>
<td>VPS28 protein homolog</td>
<td>1.5</td>
</tr>
<tr>
<td>37, 188, 193, 194, 195, 196</td>
<td>CFAB_HUMAN</td>
<td>Complement factor B precursor (Properdin factor B)</td>
<td>1.5</td>
</tr>
<tr>
<td>221</td>
<td>CSH_HUMAN</td>
<td>Chorionic somatomammotropin hormone precursor (Lactogen)</td>
<td>1.5</td>
</tr>
<tr>
<td>97, 101, 103, 104</td>
<td>SHBG_HUMAN</td>
<td>Sex hormone-binding globulin precursor</td>
<td>-1.4</td>
</tr>
<tr>
<td>76, 77, 78, 79, 227</td>
<td>APA4_HUMAN</td>
<td>Apolipoprotein A-IV precursor</td>
<td>-1.5</td>
</tr>
<tr>
<td>22, 52, 53, 54, 80, 81, 82, 125, 126, 127, 155, 156, 186, 234, 235, 239</td>
<td>CO3_HUMAN</td>
<td>Complement C3 precursor</td>
<td>-1.5</td>
</tr>
<tr>
<td>26, 28, 35, 36, 38, 39, 40, 45, 51, 135, 136, 142</td>
<td>ITH4_HUMAN</td>
<td>Inter-alpha-trypsin inhibitor heavy chain H4 precursor</td>
<td>-1.5</td>
</tr>
<tr>
<td>18, 231, 233</td>
<td>SAMP_HUMAN</td>
<td>Serum amyloid P-component precursor</td>
<td>-1.5</td>
</tr>
<tr>
<td>219, 223</td>
<td>TETN_HUMAN</td>
<td>Tetranection</td>
<td>-1.5</td>
</tr>
<tr>
<td>207, 208, 209</td>
<td>CO6_HUMAN</td>
<td>Complement component C6 precursor</td>
<td>-1.6</td>
</tr>
<tr>
<td>43</td>
<td>CENE_HUMAN</td>
<td>Centromere protein E (CENP-E-protein)</td>
<td>-1.7</td>
</tr>
<tr>
<td>56</td>
<td>CFA1_HUMAN</td>
<td>Complement factor I precursor</td>
<td>-1.8</td>
</tr>
<tr>
<td>Spot number</td>
<td>Protein symbol</td>
<td>Protein ID</td>
<td>Fold change</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------</td>
<td>------------</td>
<td>-------------</td>
</tr>
<tr>
<td>86, 89, 90, 91, 92</td>
<td>A2HS_HUMAN</td>
<td>Alpha-2-HS-glycoprotein precursor (Fetuin-A)</td>
<td>-1.8</td>
</tr>
<tr>
<td>147, 148, 149, 151, 152, 153, 154</td>
<td>APOH_HUMAN</td>
<td>Beta-2-glycoprotein I precursor (Apolipoprotein H)</td>
<td>-1.9</td>
</tr>
<tr>
<td>4, 5, 7, 17, 20, 25, 27</td>
<td>TIHY_HUMAN</td>
<td>Transthyretin precursor (Prealbumin)</td>
<td>-2.5</td>
</tr>
<tr>
<td>128, 129, 130, 131, 132</td>
<td>ANGT_HUMAN</td>
<td>Angiotensinogen precursor</td>
<td>-2.8</td>
</tr>
<tr>
<td>12</td>
<td>RETB_HUMAN</td>
<td>Plasma retinol-binding protein precursor</td>
<td>-2.8</td>
</tr>
<tr>
<td>42, 146</td>
<td>C1R_HUMAN</td>
<td>Complement C1r subcomponent precursor (EC3.4.21.41)</td>
<td>-2.9</td>
</tr>
<tr>
<td>8</td>
<td>S109_HUMAN</td>
<td>Calgranulin B</td>
<td>-3.4</td>
</tr>
<tr>
<td>237</td>
<td>PRO1_HUMAN</td>
<td>Profilin-1</td>
<td>-4.3</td>
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</tbody>
</table>
Table 13. Maternal serum placental protein levels (in ELISAs) in spontaneous pregnancies and IVF/ICSI pregnancies. Geometric mean value for each group.

<table>
<thead>
<tr>
<th>Protein (ng/mL)</th>
<th>10–12 weeks of gestation</th>
<th>18–22 weeks of gestation</th>
<th>p-value</th>
<th>10–12 weeks of gestation</th>
<th>18–22 weeks of gestation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spontaneous pregnancy</td>
<td>Placebo IVF</td>
<td>Aspirin IVF</td>
<td>Spontaneous pregnancy</td>
<td>Placebo IVF</td>
<td>Aspirin IVF</td>
</tr>
<tr>
<td>β-hCG</td>
<td>4541 (1617)</td>
<td>3844 (1667)</td>
<td>5080 (4243)</td>
<td>ns</td>
<td>976 (328)</td>
<td>962 (651)</td>
</tr>
<tr>
<td>PSG1</td>
<td>2209 (511)</td>
<td>2927 (1100)</td>
<td>3133 (1994)</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2748 (594)</td>
<td>3900 (1797)</td>
</tr>
<tr>
<td>CSH1</td>
<td>5391 (3663)</td>
<td>9176 (5909)</td>
<td>10497 (6484)</td>
<td>0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64490 (23512)</td>
<td>68444 (38073)</td>
</tr>
<tr>
<td>LBP</td>
<td>3140 (1326)</td>
<td>4268 (1603)</td>
<td>4272 (2119)</td>
<td>0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4361 (2050)</td>
<td>5257 (2271)</td>
</tr>
<tr>
<td>PAPP-A1</td>
<td>488 (497)</td>
<td>952 (1083)</td>
<td>963 (808)</td>
<td>ns</td>
<td>12652 (11545)</td>
<td>13713 (12543)</td>
</tr>
<tr>
<td>CRP</td>
<td>3729 (4795)</td>
<td>4476 (2985)</td>
<td>5019 (4760)</td>
<td>ns</td>
<td>6453 (4273)</td>
<td>5743 (3693)</td>
</tr>
<tr>
<td>VCAM1</td>
<td>269 (75)</td>
<td>294 (69)</td>
<td>274 (107)</td>
<td>ns</td>
<td>251 (46)</td>
<td>270 (78)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>812894</td>
<td>854021</td>
<td>631734</td>
<td>ns</td>
<td>569903</td>
<td>1116615</td>
</tr>
<tr>
<td>Endoglin</td>
<td>31 (6)</td>
<td>31 (8)</td>
<td>29 (7)</td>
<td>ns</td>
<td>32 (10)</td>
<td>35 (11)</td>
</tr>
<tr>
<td>Vasoerin</td>
<td>7928 (1402)</td>
<td>7991 (1153)</td>
<td>6066 (1075)</td>
<td>ns</td>
<td>6727 (1257)</td>
<td>7458 (1279)</td>
</tr>
<tr>
<td>CFD</td>
<td>2538 (601)</td>
<td>2695 (641)</td>
<td>2408 (369)</td>
<td>ns</td>
<td>2323 (581)</td>
<td>2687 (987)</td>
</tr>
</tbody>
</table>

Data are shown as mean (±SD). Values of p are from one-way ANOVA on log-transformed data. <sup>a</sup>Comparison between controls and placebo IVF/ICSI group, <sup>b</sup>comparison between controls and aspirin IVF/ICSI group.
6 Discussion

Antiplatelet drugs have an important role in the prevention of vascular events in a variety of clinical conditions, such as myocardial infarction, stroke and cardiovascular death. The use of low-dose aspirin alone (Awtry & Loscalzo 2000, Cairns et al. 2001, Antithrombotic Trialists’ Collaboration 2002, Mehta 2002) or in combination with other antiplatelet drugs (e.g. clopidogrel) (Chen et al. 2005, Dippel et al. 2010) has successfully prevented occlusive cardiovascular events and reduced mortality and morbidity in patients with ischaemic heart disease. In addition, patients with vascular risk factors such as diabetes, increased age, hypertension, obesity and adverse family history of myocardial infarction at a young age have been shown to benefit significantly from aspirin treatment (Lauer 2002, Marso 2002).

In reproductive medicine the use of anticoagulant agents tends to decrease the incidence of pre-eclampsia and IUGR (Askie et al. 2007, Duley et al. 2007) and significantly reduce the incidence of recurrent miscarriages in women with antiphospholipid antibodies (Mo et al. 2009, Mak et al. 2010). In histological examinations, all of these conditions have been shown to be associated with a thin trophoblastic shell and reduced trophoblastic infiltration into endometrial vessels and the decidua (Brosens et al. 1972, Roberts et al. 1989, Lim et al. 1997b, Sebire et al. 2002), leading to insufficient maternal spiral artery transformation and altered protein secretion of the developing placenta. In IVF and ICSI pregnancies, the incidences of early pregnancy bleeding and miscarriage (Balen et al. 1993, Koivurova et al. 2002, Ochsenkuhn et al. 2003) as well as hypertensive pregnancy complications (Maman et al. 1998, Jackson et al. 2004, Shevell et al. 2005, Allen et al. 2006) are higher than in spontaneously conceived pregnancies, suggesting altered placental development in these cases. In addition, about one third of IVF patients become pregnant after a single fresh embryo transfer and thus many patients experience recurrent implantation failure. Implantation failure after IVF and ICSI is linked to impaired uterine haemodynamics around the time of embryo transfer (Steer et al. 1992a, Coulam et al. 1994, Cacciatore et al. 1996, Zaidi et al. 1996) and higher thromboxane A2 concentrations in cultured endometrial cells (Battaglia et al. 1997) compared with cases of successful IVF/ICSI treatment.

6.1 Validation of ultrasonographic measurements

In the present study, methodological errors in Doppler ultrasonographic measurements were minimized by calculating the mean values of continuous
waveforms of at least 3 consecutive cardiac cycles and keeping the angle of insonation at less than 15 degrees (Tessler et al. 1990). However, when obtaining spiral artery blood flow velocities on the day of ET, no correction was made for the angle of insonation of the Doppler beam, since this angle cannot be determined in these small vessels. From the subendometrial region, all the vessels with high colour intensity were assessed and the waveforms with the highest Doppler-shifted frequencies were selected for further analysis. In addition, only angle-independent PI values were used to evaluate haemodynamics. In this study, all the ultrasonographic measurements were performed and analyzed by a single observer. In Study II the intra-observer variability of UtA PI measurement was 4.1% (95% CI 1.5–5.0%), which is in agreement with data in previously published studies (Bower et al. 1993, Chan et al. 1995, Weissman et al. 1995).

6.2 Validation of maternal serum proteomic analysis

Sophisticated proteomic analysis methods (Gravett et al. 2007, Nagalla et al. 2007) were used in this study. For more specific protein identification, both MALDI-TOF-MS and LC-MS/MS methods were used for ionization, analysis and fragmentation (Nesvizhskii et al. 2007). The statistical power of protein and peptide identification procedures is influenced by factors such as the discriminative ability of the database search, the size of the database and the quality of the spectra (Nesvizhskii et al. 2007). In this study, two independent search engines were used to ensure more specific protein discrimination. All ELISAs were performed in triplicate. In these analyses inter-assay and intra-assay coefficient of variation were acceptable (between 2.6 to 8.3%).

6.3 Low-dose aspirin therapy in IVF and ICSI treatment (I, V)

This randomized, placebo-controlled and double-blind study revealed that 100 mg of aspirin daily, started concomitantly with gonadotrophin stimulation for IVF and ICSI patients, did not improve pregnancy or live birth rates, the quality of oocytes/embryos, or increase ovarian responsiveness. The findings are in agreement with those of most previous studies, which have documented that 80–100 mg aspirin daily does not increase the clinical pregnancy rate compared with placebo treatment or no treatment (Urman et al. 2000, Lok et al. 2004, Moini et al. 2007, Frattarelli et al. 2008, Dirckx et al. 2009, Lambers et al. 2009a). In contrast, Rubinstein et al. (Rubinstein et al. 1999) and Waldenstrom et al. (Waldenstrom et al. 2004) showed
significantly increased pregnancy rates (Table 2) and ovarian responses in aspirin-treated women (100 and 75 mg daily, respectively) compared with placebo or no-treatment groups. However, in the study by Waldenstrom et al. (Waldenstrom et al. 2004), with a short gonadotrophin regimen, there are many aspects which make interpretation of the results difficult. Firstly, in the aspirin group the number of embryos transferred was significantly higher than in the no-treatment group, and that could have contributed to the higher pregnancy rate in the aspirin group. Secondly, the randomization method did not allow an equal possibility for every patient randomly to receive either aspirin or have no treatment. Thirdly, the study was open and not placebo-controlled, and both factors may increase the risk of study bias. A meta-analysis by Ruopp et al. (Ruopp et al. 2008) (Table 3) showed statistically significant improvement in clinical pregnancy rate in aspirin-treated women (vs. placebo). However, the open and semi-randomized study by Waldenstrom et al. (Waldenstrom et al. 2004) was included in this meta-analysis, which may have contributed to the positive result.

Data regarding the effect of low-dose aspirin therapy on oocyte and embryo quality is scarce and only a few investigators have reported these parameters. All of them found no significant differences between aspirin and placebo groups in the incidence of top-quality embryos (Dirckx et al. 2009), cumulative embryo scores (Lambers et al. 2009a) or pronuclei and cell counts of embryos on day 3 in poor responders (Frattarelli et al. 2008). In addition, all the previous studies, except for the trials carried out by Rubinstein et al. (Rubinstein et al. 1999) and Waldenstrom et al. (Waldenstrom et al. 2004) revealed similar results in the numbers of retrieved and fertilized oocytes and cleaved embryos between the study groups. These results indicate that vascular factors may not play a key role in follicle development and maturation during controlled ovarian hyperstimulation.

6.4 Effect of low-dose aspirin on uterine haemodynamics and on pregnancy outcome in IVF and ICSI patients (II, IV, V)

Doppler ultrasonographic studies have shown higher UtA vascular impedance in infertile women compared with fertile women during the late phase of the menstrual cycle, suggesting that successful implantation requires adequate uterine perfusion (Goswamy et al. 1988, Steer et al. 1994). In IVF and ICSI cycles, ultrasonographic studies have demonstrated that a substantial proportion of women with successful implantation have had more optimal uterine haemodynamics (Noyes et al. 1995, Battaglia et al. 1997) compared with women without conception. In particular,
increased UtA vascular impedance (Coulam et al. 1994, Cacciatore et al. 1996, Zaidi et al. 1996, Battaglia et al. 1997) and low subendometrial or endometrial blood flow (Zaidi et al. 1995, Chien et al. 2002) have been associated with poor implantation and low pregnancy rates. Rubinstein et al. (Rubinstein et al. 1999) reported significantly lower UtA PI values on the day of hCG administration and a higher pregnancy rate in aspirin-treated women (100 mg/day) with tubal aetiology compared with a placebo group. On the other hand, in studies by Lok et al. (Lok et al. 2004) with unselected poor IVF-responders and by Lambers et al. (Lambers et al. 2009a) with non-tubal factor IVF patients with previous implantation failure, no significant differences were found in UtA PI values or pregnancy rates between the aspirin (80 mg/day and 100 mg/day, respectively) and placebo groups. A recent meta-analysis by Khairy et al. (Khairy et al. 2007), which included the studies by Rubinstein et al. (Rubinstein et al. 1999) and Lok et al. (Lok et al. 2004), showed a decrease in UtA PI values in aspirin-treated women. In the present study, mean PI values in the uterine, arcuate, radial and spiral arteries were comparable between the groups, demonstrating that among unselected IVF and ICSI patients low-dose aspirin therapy does not affect uterine vascular impedance during a long gonadotrophin regimen. In addition, our results showed that examination of UtA blood flow velocity waveforms on the day of embryo transfer is not useful in prediction of implantation success or pregnancy outcome.

As a secondary outcome measure in the present study, the incidence of non-optimal uterine haemodynamics (bilateral UtA PI value ≥3.0) was lower among the aspirin-treated women than in the placebo group. Some previous studies have shown that UtA PI values of ≥3.0 at the time of embryo transfer predict poor outcome in IVF and ICSI treatment (Steer et al. 1992a, Coulam et al. 1994, Cacciatore et al. 1996, Zaidi et al. 1996). Kuo et al. (Kuo et al. 1997) found a significant improvement in uterine haemodynamics during the peri-implantation period among women with unexplained infertility whose UtA PI value was ≥3.0 during the previous natural menstrual cycle and who were treated with low-dose aspirin during subsequent cycles. Furthermore, endometrial cell cultures have shown significantly lower thromboxane A₂ levels in women with conception cycles after controlled ovarian hyperstimulation compared with women with non-conception cycles (Battaglia et al. 1997). Even though none of the patients with increased uterine artery vascular impedance experienced pregnancy in the present study, the results of previous studies suggest that implantation can occur in the presence of non-optimal uterine haemodynamics at the time of embryo transfer (Isaksson et al. 2003).

In women taking low-dose aspirin who became pregnant after IVF or ICSI
treatment, arcuate artery PI values at 6+ gestational weeks and UtA PI values at 18+ gestational weeks were significantly lower than in the placebo group. Increased UtA vascular impedance during the second trimester increases the risk of pre-eclampsia and other hypertensive pregnancy complications (Albaiges et al. 2000, Papageorghiou et al. 2001). Histological examinations of pre-eclamptic placentas have shown shallow trophoblast invasion into the maternal spiral arteries, hampering their normal physiological transformation to slack and non-occlusive sacks. Normal transformation of spiral arteries allows efficient blood flow into the intervillous space and further leads to physiological high-volume and low-resistance uteroplacental circulation. In pre-eclampsia, spiral arteries maintain their occlusive properties, which decrease blood supply to the placenta during pregnancy. In addition, in pre-eclampsia production of thromboxane A\(_2\), which induces platelet aggregation and constriction of vascular smooth muscle, is excessive (Wang et al. 1992). Low-dose aspirin irreversibly inhibits COX-1 activity, leading to decreased synthesis of thromboxane A\(_2\) (Vane 1971, Willis 1974), thus at least to some extent correcting the imbalance between thromboxane and prostacyclin production. The results of the present study may reflect more successful trophoblastic invasion with improved remodelling of spiral arteries in aspirin-treated subjects compared with the placebo group. Moreover, the incidence of bilateral UtA notches at 18+ gestational weeks tended to be lower in the aspirin group compared with the placebo group, indicating that low-dose aspirin therapy when started prior to pregnancy may improve uteroplacental circulation. On the other hand, in Study IV, UtA PI values were within normal limits in every woman who developed pre-eclampsia (late onset; > 34 weeks) and none of them had bilateral UtA notches at 18+ gestational weeks. This demonstrates that pre-eclampsia may develop even in the presence of normal uteroplacental circulation at mid-gestation.

In this study, the incidence of pre-eclampsia classified according to ACOG (2002) criteria was somewhat higher (13.9% in Study IV and 7.5% in Study V) than in the general pregnant population (2–7%), which is in agreement with previous findings in IVF and ICSI pregnancies (Shevell et al. 2005). There was no statistically significant reduction in the incidence of pregnancy-related hypertensive complications in the low-dose aspirin group. This finding is in agreement with the results of large randomized controlled trials, which have shown that antiplatelet therapy, mainly low-dose aspirin (60–100 mg/day), when started beyond 12 weeks of gestation, has no significant effect on the incidence of pre-eclampsia and IUGR, even in a high-risk population (CLASP 1994, ECPPA 1996, Rotchell et al. 1998, Harrington et al. 2000). In these studies the lack of significant reduction in the
incidence of pre-eclampsia and IUGR may have been due to the fact that low-dose aspirin therapy was started too late, when the primary pathophysiological insult leading to clinical manifestation of pre-eclampsia had already occurred. This is supported by findings in some small studies in which a subgroup of women with abnormal UtA vascular impedance at 12–16 gestational weeks (Vainio et al. 2002, Ebrashy et al. 2005), or women at a high risk of pre-eclampsia based on risk algorithm assessment at 11–14 gestational weeks (Baschat et al. 2009) have shown a significant reduction in the incidences of PIH, pre-eclampsia or IUGR when treated with low-dose aspirin. Furthermore, the results of a recent meta-analysis by Bujold et al. (Bujold et al. 2010) revealed that low-dose aspirin therapy initiated before 16 weeks of gestation was associated with a significant decrease in the incidence of pre-eclampsia, severe pre-eclampsia, IUGR and preterm birth in women identified to be at risk of pre-eclampsia, whereas aspirin started beyond 16 weeks’ had no effect on the outcome parameters.

The study by Lambers et al. (Lambers et al. 2009b) was the first to reveal a significant reduction in the incidence of hypertensive pregnancy complications in aspirin-treated (100 mg/day) versus placebo-treated non-tubal-factor IVF and ICSI patients with previous implantation failure, when medication was started prior to conception. Our results do not support this finding. Different study populations in the study by Lambers et al. (Lambers et al. 2009b) and in the present study could partially explain the different results. Women with tubal factor infertility were excluded from the study by Lambers et al. (Lambers et al. 2009a, 2009b) and all the patients had experienced at least one previous conception failure. Infertility of tubal aetiology is generally associated with good outcome in IVF and ICSI treatments. In the present study, nearly 60% of the patients had their first ovarian stimulation and all forms of aetiology of infertility were included. However, the rates of clinical pregnancy and live birth, as well as the outcome parameters of IVF/ICSI treatment were comparable with those in the study by Lambers et al. (Lambers et al. 2009a). In their study (Lambers et al. 2009b) the incidence of hypertensive pregnancy complications in the placebo group was 26.9%, which seems to be exceptionally high. In large randomized clinical trials among high-risk populations, the incidence of pre-eclampsia has varied between 2.5–8.7% in the placebo group (CLASP 1994, ECPPA 1996, Harrington et al. 2000). In pregnancies after IVF and ICSI, the incidence of hypertensive pregnancy complications has been shown to be increased (2- to 3-fold) compared with that in spontaneous pregnancies, even after adjusting for maternal age, parity and multiple gestation (Maman et al. 1998, Jackson et al. 2004, Shevell et al. 2005, Allen et al. 2006).
In the present study the incidence of hypertensive pregnancy complications was 18.2% in the placebo group, being comparable with that in previous studies among IVF and ICSI patients. Another explanation for the high incidence of hypertensive pregnancy complications in the study by Lambers et al. (Lambers et al. 2009b) could be the relatively high proportion of twin pregnancies in the placebo group. However, on the basis of our results, this explanation seems to be unlikely.

6.5 Maternal serum proteomic profile (III)

6.5.1 Placental proteins in spontaneous and IVF pregnancies

A total of 368 maternal serum proteins were identified that showed significantly different concentrations in the spontaneous pregnancy and placebo IVF/ICSI groups. Protein expression differences were noted in extra-cellular matrix, cytoskeletal, vascular, immune and transport proteins, all of them important in the maintenance of pregnancy (Das et al. 2002, Wulff et al. 2003, Bulmer et al. 2010). The present results are in accordance with those in several previous studies that have shown alterations in maternal serum placental protein concentrations in IVF/ICSI pregnancies when compared with spontaneously conceived pregnancies during the first half of gestation (Liao et al. 2001, Perheentupa et al. 2002, Hui et al. 2005, Tul & Novak-Antolic 2006, Anckaert et al. 2008). It has been postulated that high doses of exogenous hormones used in ovarian hyperstimulation and multiple corpora lutea could affect maternal serum protein levels (Heinonen et al. 1996, Liao et al. 2001). However, a study by Perheentupa et al. (Perheentupa et al. 2002) showed that maternal serum β-hCG levels in the second trimester were also elevated in unstimulated frozen-thawed embryo cycles, suggesting that the ovarian superovulatory therapy and IVF itself are not the primary causes. Recently, Ranta et al. (Ranta et al. 2010) demonstrated that subfertile women whose time-to-pregnancy interval was over two years had alterations in maternal serum protein levels that were similar to those in IVF pregnancies. This suggests that maternal serum protein changes could be related to subfertility and infertility rather than to the use of ovarian hyperstimulation techniques, or they could be a sign of delayed placental maturation, as seen in pregnancies with chromosomal abnormalities.

At the end of the first trimester, maternal serum concentrations of PSG1, CSH1 and LBP were significantly increased in the placebo IVF/ICSI group compared with the control group. However, at 18–22 gestational weeks only PSG1 remained increased. It has been shown that in IVF/ICSI pregnancies, maternal serum CSH1
and PSG1 concentrations are lower than in spontaneous pregnancies early in the first trimester (<10 gestational weeks). However, at 12–14 gestational weeks, their concentrations were higher in IVF/ICSI pregnancies than in the spontaneous pregnancies, and PSG1 concentrations also remained elevated at 17 gestational weeks (Bersinger et al. 2004). These findings are in agreement with the present results. CSH1 and PSG1 are produced by the trophoblast cells soon after fertilization (Dimitriadou et al. 1992) and secreted into the maternal blood, with increasing concentrations during pregnancy as placental weight increases (Pedersen et al. 1995). CSH1 mimics the actions of growth hormone and prolactin, stimulating the synthesis of IGF I (Vatten et al. 2002). In early pregnancy, low maternal serum CSH1 concentrations in IVF/ICSI pregnancies could be a sign of slow trophoblast cell proliferation. However, after 10 gestational weeks, CSH1 secretion seems to increase prior to that of PAPP-A, which also regulates IGFs by cleaving insulin-like growth factor-binding protein 4 (Bersinger et al. 2004). The exact physiological role of PSG1 is unknown. It has been proposed that pregnancy-specific factors could inhibit T-cell function and shift the balance from Th1-type to Th2-type reactivity (Motran et al. 2003). Moreover, PSGs induce TGFβ1, which regulates VEGF and PlGF in the developing placenta (Sanchez-Elsner et al. 2001, Jeon et al. 2007). In IVF/ICSI pregnancies, PSG1 may significantly promote angiogenesis during the late first and the second trimester.

6.5.2 Effect of low-dose aspirin on placental proteomics in IVF pregnancies

In the aspirin IVF/ICSI group, maternal serum CSH1 concentrations were increased at 10–12 gestational weeks, and VCAM1 concentrations at 18–22 gestational weeks compared with the control group. In addition, in the aspirin and placebo IVF/ICSI groups, several differently expressed maternal serum placental proteins, including extra-cellular matrix, complement and transport proteins were found. These results indicate that low-dose aspirin can modify the early placental process. The placenta produces several adhesion molecules, including VCAM1, which mediate leukocyte adhesion and extravasation (Jaakkola et al. 2000). It seems that VCAM1 plays an important role in early placentation and embryo development (Rajashekar et al. 2003). Furthermore, in pregnancies complicated by IUGR, the expression of VCAM1 is decreased (Rajashekar et al. 2003). By binding to integrin α4 receptors on the surface of T-cells, VCAM1-expressing tumour cells can cause immune cell migration away from them and promote their survival. If
tumour cells do not express VCAM1, there is no interaction with integrin α4 in the immune cells and this leads to tumour cell death (Wu 2007). The fetus inherits paternal histocompatibility antigens and therefore trophoblast cells act partly like tumour cells. Expression of placental VCAM1 and its interaction with integrin α4 is necessary for normal fusion of the chorioallantois. In addition, it promotes efficient development of the placental and fetal vascular system (Yang et al. 1995).

Decreased PAPP-A and increased inhibin-A concentrations in the first trimester may reflect poor placentation and result in increased risks of pre-eclampsia, IUGR and stillbirth later in pregnancy (Sebire et al. 2000, Tul et al. 2003). Furthermore, in pre-eclampsia intravascular thromboxane production is excessive, which induces vasoconstriction and platelet aggregation (Wang et al. 1992). By inhibiting vasoconstriction and platelet aggregation, low-dose aspirin may improve placentation, normalizing placental protein production and reducing the risk of pre-eclampsia. Decreased thromboxane production during implantation improves pregnancy rates after IVF, suggesting that the prostacyclin-thromboxane balance contributes to endometrial formation and embryo implantation (Battaglia et al. 1997). In the present study, via proteomic analysis, we identified a distinct protein profile in aspirin-treated versus placebo-treated IVF/ICSI women during the first half of pregnancy. A cluster of matrix, acute phase and immunomodulating proteins, as well as alpha-1-antichymotrypsin, phosphatidylinositol-glycan-specific phospholipase D, leucine-rich alpha-2-glycoprotein and complement factor H were more abundant, whereas calgranulin and profiling-1 were less abundant in the aspirin-treated than in the placebo-treated IVF/ICSI women. The over-expressed proteins in the aspirin group are known to be involved in tissue protection (by inactivating proteolytic enzymes (Kalsheker 1996)), signal transduction and in cell development, differentiation and adhesion (O’Donnell et al. 2002).

Maternal serum vasorin concentrations decreased significantly in spontaneous pregnancies and in the placebo IVF/ICSI group during the first half of pregnancy. However, in the aspirin group, vasorin levels remained unchanged. Vasorin acts as a binding protein of TGFβ, and low concentrations of vasorin result in elevated concentrations of circulating TGFβ, which promote trophoblast differentiation and inhibit invasion into the maternal spiral arteries (Caniggia et al. 1999, Ikeda et al. 2004). Vasorin is predominantly expressed in vascular smooth muscle and its expression has been shown to be down-regulated during vessel repair after arterial injury (Ikeda et al. 2004). Low-dose aspirin modulates pathological stimuli in the vessel wall by preventing platelet aggregation in the sites of neointimal formation, and may thus prevent down-regulation of vasorin. In addition, maternal serum
concentrations of immune proteins were not affected by low-dose aspirin therapy. Increased CRP levels during pregnancy have been linked to an increased risk of pre-eclampsia (Derzsy et al. 2010).

6.6 Factors affecting the efficacy of low-dose aspirin therapy

6.6.1 Dosage

A single oral dose of 6 to 100 mg of aspirin results in dose-dependent inhibition of platelet COX activity, with 100 mg almost completely suppressing the synthesis of thromboxane \( A_2 \) in normal subjects (Patrignani et al. 1982). However, daily administration of 30 to 50 mg of aspirin results in virtually complete suppression of platelet thromboxane \( A_2 \) synthesis after 7 to 10 days (Patrono 1994). Aspirin doses of 80–160 mg daily in healthy volunteers (Cerletti et al. 2003) and 0.5–2.0 mg/kg daily in hypertensive pregnant women (Vainio et al. 1999) have been shown to increase the prostacyclin-thromboxane ratio. Viinikka et al. (Viinikka et al. 1993) noted that an aspirin dose of 50 mg/day inhibited more than 90% of platelet thromboxane \( A_2 \) production, and significantly decreased the urinary excretion of thromboxane \( A_2 \) metabolites, but did not decrease the urinary excretion of prostacyclin metabolites. Although very low doses of aspirin effectively prevent systemic platelet thromboxane \( A_2 \) synthesis, there is evidence that aspirin at doses of <80 mg/day does not affect placental thromboxane \( A_2 \) excretion (Hauth et al. 1995b, Wang et al. 1996, Dumont et al. 1999). Based on previous data, chronic administration of 100 mg of aspirin daily should be sufficient to increase the prostacyclin-thromboxane ratio in unselected infertile women undergoing controlled ovarian hyperstimulation, even taking into consideration the weight gain during pregnancy.

6.6.2 Aspirin resistance

Aspirin reduces the risk of cardiovascular events by 25% in a broad category of patients with arterial vascular disease (Antiplatelet Trialists’ Collaboration 1994). However, its effectiveness is limited because 10 to 20% of patients with arterial vascular disease and aspirin therapy have a recurrent vascular event during long-term follow-up (Patrono 2001). The results of several large randomized trials concerning the effects of low-dose aspirin on prevention of pre-eclampsia and IUGR have been disappointing (CLASP 1994, ECPPA 1996, Harrington et al. 2000). Moreover, the studies have failed to show any clear beneficial effect
of the use of low-dose aspirin therapy in IVF and ICSI patients, who may have suboptimal uterine haemodynamic environments for implantation and an increased risk of hypertensive pregnancy (Lok et al. 2004, Lambers et al. 2009a, 2009b), even though theoretically aspirin therapy could improve uterine blood flow and perfusion in these women. There are several possible explanations for the limited efficacy of aspirin. First, it is well recognized that platelets can be activated by pathways that are not blocked by aspirin (Valles et al. 1998, Santos et al. 2000). In addition, it has been suggested that higher doses of aspirin than are currently used (30 to 325 mg/day) may be required in some patients to achieve an optimal antithrombotic effect (Patrono 1998, Dyken et al. 1992). Finally, some patients may be able to generate thromboxane A₂ despite normal therapeutic doses of aspirin and therefore fail to benefit from aspirin treatment (Vejar et al. 1990). None of the studies on IVF and ICSI patients have involved measurement of urinary excretion of thromboxane A₂ metabolites or analysis of platelet activity during low-dose aspirin therapy. On the other hand, it may be that pregnancy failure is not dependent on uterine and ovarian blood flow or perfusion.

6.6.3 Time-dependent differences in low-dose aspirin administration

Aspirin selectively decreases blood pressure as a function of duration of administration in relation to the rest-activity cycle of each individual woman. This phenomenon has been shown in healthy volunteers, in patients with mild hypertension as well as in pregnant women at an increased risk of pregnancy-induced hypertension or pre-eclampsia in randomized and placebo-controlled studies carried out by Hermida et al. (Hermida et al. 1997a, 1997b, 1997c, 1999). In the study with pregnant women the researchers administered 100 mg aspirin orally in one dose starting at 12 to 16 weeks of gestation at different times of day. When low-dose aspirin was administered at the time of waking it did not affect blood pressure (versus placebo). When low-dose aspirin was administered 8 hours after waking, blood pressure reduction was statistically significant, and it was even more so when administered at bedtime (Hermida et al. 1997b). The mechanism is unclear, but it is assumed that the effect is linked to circadian rhythms of thromboxane A₂ and prostacyclin production (Haus et al. 1990), circulating angiotensin II (Beilin et al. 1982) and angiotensin sensitivity in pregnancy (Delemarre et al. 1996).
7 Limitations of the study

No systematic placental histological examination was performed to investigate the effect of low-dose aspirin therapy on developing placentas in IVF and ICSI patients when medication was initiated prior to pregnancy. Hypertensive pregnancy complications have been characterized by superficial trophoblast invasion into the uterine spiral arteries (Brosens et al. 1972; Meekins et al. 1994) and obliteration of small muscular arterioles in tertiary villi (Giles et al. 1985). However, in a previous study among women at an increased risk of pre-eclampsia, placental histology did not differ significantly in women treated with low-dose aspirin (80 mg/day) versus no treatment (Tarim et al. 2006).

In Studies II and IV, the sample sizes were limited. However, the sample size in Study IV was sufficient to show statistically significant differences in uteroplacental haemodynamic parameters between the groups. On the basis of the results of the present work (II, IV, V) we cannot definitively conclude whether or not low-dose aspirin therapy decreases the incidence of pre-eclampsia or delays the onset of clinical pre-eclampsia or improves other pregnancy outcome parameters in IVF and ICSI pregnancies.
8 **Clinical implications**

According to the results of this work, routine use of low-dose aspirin therapy cannot be recommended among unselected IVF/ICSI women to improve the clinical outcome. However, aspirin reduces the incidence of non-optimal uterine artery haemodynamics on the day of ET, which has been shown to impede embryo implantation and decrease the pregnancy rate.

Women on low-dose aspirin therapy showed different placental protein expression (detected by maternal serum proteomics) and reduced uteroplacental vascular impedance at the end of the first half of pregnancy compared with placebo-treated women. However, low-dose aspirin therapy did not decrease the incidence of hypertensive pregnancy complications in unselected IVF and ICSI patients. This demonstrates that pre-eclampsia may develop in the presence of normal uteroplacental circulation in mid-gestation and supports the hypothesis that clinical pre-eclampsia is a heterogeneous disease with multiple aetiological factors. It would be relevant to revisit the term pre-eclampsia and perceive early- and late-onset pre-eclampsia as two different conditions. Even if abnormal placental circulation is a fundamental factor in the pathogenesis of pre-eclampsia, it seems not to be the only or the most significant pathophysiological insult in late-onset pre-eclampsia. However, there could still be some subgroups of patients who are prone to early-onset pre-eclampsia and they could benefit significantly from low-dose aspirin treatment when started prior to implantation.

Placental protein expression in IVF/ICSI pregnancies is different from that in spontaneous pregnancies, but the aetiology of this unique protein pattern is unknown. In addition, low-dose aspirin therapy modifies the placentation process and seems to normalize the maternal serum proteome profile towards that found in spontaneous pregnancies. Complete characterization of differentially expressed proteins in placentas in women treated with low-dose aspirin is important. In the present work, only those proteins for which ELISAs were available commercially could be quantitatively analysed. New proteome techniques, such as functional proteome analysis, and increased availability of commercial antibodies for proteins may help us to understand the effects of low-dose aspirin and other remedies in the developing placenta and help in the prevention of pathological pregnancy conditions.
9 Conclusions

1. Low-dose aspirin therapy started prior to conception does not improve ovarian responsiveness, pregnancy outcome (I) or uterine receptivity (II) in unselected subjects undergoing IVF/ICSI treatment. The number of oocytes, the number and quality of embryos, the incidence of clinical pregnancy, and endometrial thickness on the day of embryo transfer were comparable in the aspirin and placebo groups.

2. Uterine artery vascular impedance on the day of embryo transfer did not differ between the study groups. However, the incidence of bilateral UtA PI values of $\geq 3.0$ (non-optimal uterine haemodynamics) on the day of ET was significantly lower in the aspirin group than in the placebo group (II).

3. In IVF pregnancies the maternal serum placental protein profile is different from that in spontaneous pregnancies during the first half of pregnancy, especially in the first trimester. Low-dose aspirin therapy modifies placentation, decreasing the expression of placental proteins that inhibit trophoblast invasion and promote adverse immune responses, and increasing the expression of adhesion and proteolytic proteins (III).

4. During early pregnancy, the arcuate artery pulsatility index, and in mid-pregnancy, the UtA pulsatility index were lower in the aspirin group than in the placebo group (IV). Despite improved uterine haemodynamics during the first half of pregnancy in the aspirin group, the incidence of hypertensive pregnancy complications was comparable in the two groups (V).
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